Anogenital distance as a biomarker for incomplete masculinization

Molecular mechanisms in the perineum

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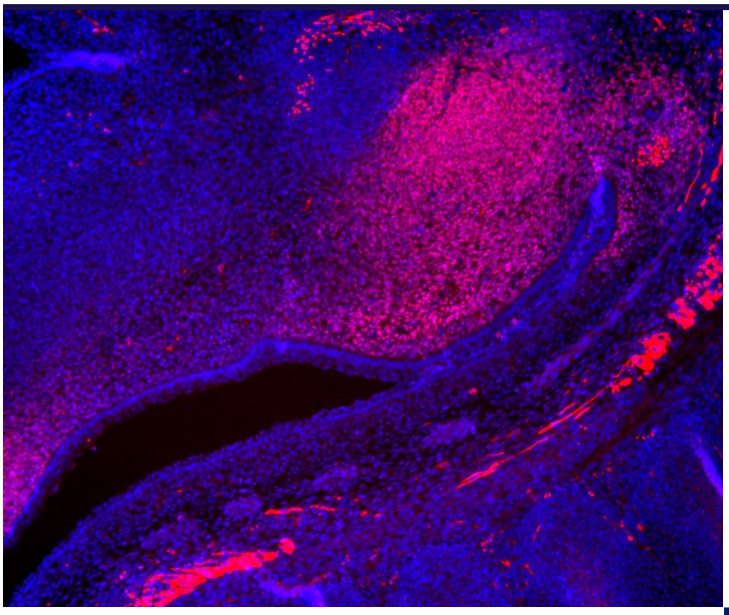

PhD Thesis

Camilla Lindgren Bech
November 2020

National Food Institute
Technical University of Denmark

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Molecular mechanisms in the perineum

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PhD thesis

Research Group of Molecular and Reproductive Toxicology
Division of Diet, Disease Prevention and Toxicology
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Thesis title

Anogenital distance as a biomarker for incomplete masculinization

Molecular mechanisms in the perineum

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Preface

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November 18th, 2020

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Summary

In both humans and rodents, development of the male reproductive organs and general masculinization of the fetus depends on androgen signaling. This signaling needs to take place within a short time window of fetal life, referred to as the masculinization programming window (MPW). In many respects, androgen action in this time window lays the foundation for male reproductive health later in life. Fetal androgen action also stimulates the growth of the perineal muscles and thereby influence the length of the perineum, i.e. the anogenital distance (AGD). This distance, from the anus to the external genitalia, is directly related to fetal androgen action and is twice as long in males as in females. Consequently, a short male AGD at birth is considered a unique biomarker of disrupted fetal androgen action in both rodents and humans. A short AGD is associated with adverse effects on male reproductive health such as cryptorchidism and hypospadias at birth as well as poor sperm quality and reduced fertility in adulthood. This morphometric measure is therefore used in a regulatory context to test chemicals for their endocrine disruptive properties.

Although we know that androgen signaling is critical for perineal growth, our mechanistic knowledge remains limited. In addition, some chemicals affect AGD in ways that cannot be predicted based on their anti-androgenic potential *in vitro*. This suggests that other regulatory pathways also play a role in determining the AGD outcome, at least as modifiers of the androgen-mediated effect. Knowledge of the regulatory pathways and molecular events that control perineal development is essential when extrapolating from rodent toxicity studies to humans. In addition, such knowledge could contribute to future work on the development of adverse outcome pathways (AOPs) and new, refined non-animal test strategies in chemical risk assessment. The aim of this PhD project was therefore to identify potential additional signaling pathways affected in the perineal tissues of males with short AGD.

An extensive literature search was conducted to collate the available literature pertaining to the relationship between xenobiotic exposure and effects on the AGD. For the experimental work, two different approaches were used. First, *in utero* exposure to known anti-androgenic compounds was used to induce short AGD in the male offspring and compare the transcriptional profile of the perineum to that of the control males and the control females. Second, perineum from male and female offspring at different developmental stages was used to investigate the mechanisms that govern the normal, sex-specific development of the perineum.

In the first study, *in utero* exposure to the 5α-reductase inhibitor finasteride was used to induce a short AGD in the male fetuses at gestational day 21. Microarray analysis of the perineum revealed that the control males, control females and exposed males each had their distinct transcriptional profile. Importantly, the transcriptional profile of the perineum from the exposed males was more closely related to the control females than the control males. This confirms that the short, feminized male AGD is associated with a feminized transcriptional profile of the perineal tissues. Four genes related to Wnt and estrogen signaling (*Wnt2, Sfrp4, Esr1,* and *Padi2*) had a sexually dimorphic expression pattern and expression of the estrogen related factors (*Esr1* and *Padi2*) were affected in the finasteride exposed males. These pathways could therefore play a role in the sex-specific development of the perineal tissues. The identification of the four Wnt and estrogen related factors provided targets for further studies on the molecular events governing perineal development.

The second study investigated if the Wnt and estrogen related factors identified in the first study were affected by exposure to other anti-androgenic chemicals. The expression of these factors in the perineum was analyzed following *in utero* exposure to enzalutamide, vinclozolin and procymidone. Out of these three compounds, the androgen receptor antagonist enzalutamide had the most marked effect on fetal AGD and also had significant effect on perineal expression of *Sfrp4.* For vinclozolin and procymidone, two pesticides with known anti-androgenic properties, smaller effects were seen on AGD at the doses used but no statistically significant effects on perineal expression *Wnt2, Sfrp4, Esr1,* and *Padi2*. These results, combined with the knowledge from the first study with finasteride, suggest that substantial effects on AGD are necessary to detect effects at the transcript level in the perineal tissues. However, all the tested anti-androgenic compounds showed indications of a similar mode of action on the perineum, although this could not be proven by statistical methods due to a low power and/or large biological variation. Together with the available literature, the results from the first two studies could suggest that Wnt and estrogen signaling may modulate AGD outcome by regulating cell proliferation and differentiation in the fetal perineal tissues.

The last study focused on the normal, sexually dimorphic development of the perineum. Here, gene expression was investigated in perineal tissues from male and female fetuses at different stages of development. The results suggest that there is a male-specific increase in non-myocytic progenitor cells in the perineum during early development. These cells provide the basis for differentiation into muscle cells and thus a male-specific increase in myogenic cells at later stages of fetal development. These are very preliminary data and should be followed up by larger, more detailed studies using techniques suited for tracking cell populations in a spatiotemporal context.

Collectively, the studies of this PhD project show that the sexually dimorphic AGD is associated with a sexually dimorphic transcriptional profile of the perineum. Furthermore, anti-androgenic chemicals that induce short male AGD induce a concomitant feminization of the perineal transcriptional profile. The Wnt and estrogen signaling pathways provide targets for future research in perineal development as they are expressed in a sexually dimorphic pattern and are affected in the perineum of males with short AGD following exposure to potent anti-androgens. In addition, the present findings suggest that the sex-specific size difference of the perineal muscles may be based on differences in progenitor cell populations and their subsequent differentiation into muscle cells.

This PhD thesis identifies new possible signaling pathways that may contribute to the development of the perineum and AGD. It highlights possible targets and new approaches to be used in future investigations that may ultimately contribute to describing the detailed adverse outcome pathway (AOP) for chemically induced effects on AGD.

Sammendrag

Udviklingen af hanners kønsorganer og den generelle maskulinisering af fosteret er både hos mennesker og i gnavere afhængig af signalering af androgener. Denne signalering foregår i et kortvarigt tidsrum under fosterudviklingen kaldet "programmeringsvinduet for maskulinisering". Det er i høj grad den androgene aktivitet i dette tidsrum, der danner grundlaget for hannernes reproduktive sundhed senere i livet. Aktiviteten af androgener hos fosteret inducerer desuden vækst af de muskler der er i mellemkødet og påvirker derved længden af mellemkødet også kaldet den anogenitale afstand (AGD). Denne afstand er således direkte relateret til den androgene aktivitet der har været i fostertilstanden og afstanden er normalt dobbelt så lang i hanner som hos hunner. Derfor betragtes en kort AGD i hanner ved fødslen som et tegn på en forstyrret androgen signalering i fostertilstanden. Kort AGD er associeret med negative effekter på den reproduktive sundhed i hanner såsom kryptorkisme og hypospadi ved fødslen samt lav sædkvalitet og lav fertilitet i voksenlivet. AGD undersøges derfor når kemikalier testes for hormonforstyrrende effekter i studier af reproduktionstoksicitet.

Selvom vi ved, at androgen signalering er nødvendig for udviklingen af mellemkødet, er vores forståelse af mekanismerne involveret i denne udvikling begrænset. Ydermere er der tilfælde hvor kemikalier påvirker AGD på måder, der ikke kan forudses baseret på deres anti-androgene aktivitet i *in vitro* studier. Dette kan være et tegn på at også andre signaleringsveje kan påvirke AGD, i det mindste, ved at ændre den androgen-medierede effekt. Kendskabet til sådanne signaleringsveje og de molekylære mekanismer, der er involveret i udviklingen af mellemkødet, er vigtigt, når vi skal overføre resultater fra toksikologiske studier i rotter, til viden om effekter i mennesker. Denne viden kan i fremtiden bidrage til udviklingen af nye og forbedrede metoder til risikovurderingen af kemikalier, uden brug af forsøgsdyr. Det overordnede formål med dette Ph.d. projekt er at identificere andre mulige signaleringsveje, der kan være påvirket i mellemkødet fra hanrotter med kort AGD.

En grundig litteratursøgning dannede basis for en sammenskrivning af den aktuelle viden om forholdet mellem eksponering for kemikalier og deres effekter på AGD. I forbindelse med det eksperimentelle arbejde i denne Ph.d., blev der brugt to forskellige fremgangsmåder. Først blev rottefostre eksponeret for kendte anti-androgene kemikalier via moderen for at inducere kort AGD i hannerne og derefter sammenligne mellemkødets transkriptionelle profil med den profil der er i kontrolhanner og kontrolhunner. Dernæst blev mellemkødet isoleret fra han- og hunfostre i forskellige udviklingsstadier. Disse blev brugt til at undersøge de mekanismer der styrer den normale, kønsspecifikke udvikling af mellemkødet.

I det første studie blev kort AGD induceret i hanfostrene ved at eksponere den drægtige hun for finasterid, der er en 5α-reduktase-hæmmer. Microarray-analyse viste at kontrolhanner,

kontrolhunner og eksponerede hanner har hver deres individuelle transkriptionelle profil. Af særlig betydning var, at den transkriptionelle profil for de eksponerede hanner var tættere på kontrolhunnernes end kontrolhannernes. Dette bekræfter at kort, feminiseret AGD i hanner er forbundet med en feminiseret transkriptionel profil af mellemkødet. Fire gener alle relateret til Wnt og østrogen signaleringen (*Wnt2, Sfrp4, Esr1,* and *Padi2*) var udtrykt kønsspecifikt og genekspressionen af de østrogen relaterede faktorer (*Esr1,* and *Padi2*) var påvirket i de finasterid eksponerede hanner. Dette kunne tyde på at disse signaleringsveje spiller en rolle i den kønsspecifikke udvikling af mellemkødet og dermed AGD. Identifikationen af disse Wnt og østrogenrelaterede faktorer er derfor gode kandidater, hvis man i fremtidige studier vil undersøge de molekylære mekanismer, der styrer udvikling af mellemkødet nærmere.

I det næste studie, blev det undersøgt hvorvidt de Wnt og østrogenrelaterede faktorer, identificeret i det første studie, også blev påvirket af andre anti-androgene kemikalier. Genekspression af disse faktorer i mellemkødet blev analyseret efter eksponering af fostrene via moderen for stofferne enzalutamid, vinclozolin og procymidon. Ud af disse tre kemikalier havde androgen receptor antagonisten enzalutamid den største effekt på fostrenes AGD og påvirkede også ekspressionen af *Sfrp4* i mellemkødet. Vinclozolin og procymidon, to kemikalier med kendte anti-androgene egenskaber, inducerede mindre effekter på AGD ved de aktuelle doser og de påvirkede ikke mellemkødets genekspression af de fire testede gener. Disse resultater, sammen med resultaterne for finasterid i det første studie, tyder på at det er nødvendigt med en betydelig påvirkning af AGD for at påvise forskelle på transkript niveau i mellemkødet. Dog viste alle de testede anti-androgene stoffer indikationer på samme virkningsmekanisme i mellemkødet selvom dette ikke kunne påvises med statistiske metoder på grund af den lave statistiske power og/eller den store biologiske variation. Sammen med den tilgængelige litteratur på området kunne resultaterne fra disse første forsøg tyde på at Wnt og østrogen signalering påvirker effekten på AGD ved at regulere celle proliferation og differentiering i mellemkødet under foster tilstanden.

Det sidste studie fokuserede på den normale, kønsspecifikke udvikling af mellemkødet. Her blev genekspressionen undersøgt i mellemkødet fra ikke-eksponerede han- og hunfostre på forskellige stadier af fosterudviklingen. Resultaterne tyder på at der er en hanspecifik øgning i "non-myocytiske progenitor" celler i mellemkødet under de tidlige stadier af udviklingen. Disse celler danner grundlaget for differentieringen af muskelceller og derfor også for en hanspecifik øgning i myocytter (muskelceller) under senere stadier af udviklingen. Disse data er dog præliminære og bør opfølges med flere større, detaljerede studier og teknikker der er velegnede til at følge cellepopulationer over både tid og sted.

Tilsammen viser studierne i dette Ph.d. projekt at den kønsspecifikke AGD er forbundet med en kønsspecifik transkriptionel profil af mellemkødet. Ydermere ses det, at kemikalier der inducerer en kort AGD i hanner samtidigt inducerer en feminiseret transkriptionel profil af mellemkødet. Wnt- og østrogen-signaleringsvejene udgør gode kandidater for fremtidige studier i mellemkødets udvikling. Dette fordi de er udtrykt kønsspecifikt og er påvirket i mellemkødet fra hanner med kort AGD, som følge af eksponering for potente anti-androgener. Resultaterne antyder ydermere at den kønsspecifikke størrelsesforskel i mellemkødet muligvis kan skyldes forskelle i progenitor-cellepopulationer og deres efterfølgende differentiering til muskelceller.

Denne Ph.D. afhandling identificerer nye mulige signaleringsveje der måske kan påvirke udviklingen af mellemkødet og AGD. Afhandlingen fremhæver mulige molekylære kandidater og nye metoder der eventuelt kan bruges i fremtidig forskning, som ønsker at beskrive de detaljerede molekylære mekanismer, der leder til effekter på AGD efter eksponering for kemikalier.

List of manuscripts

Manuscripts and published papers included in this PhD thesis

Manuscript I

Schwartz C. L., Christiansen S., Vinggaard AM., Axelstad M., Hass U., Svingen T. (2018) Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Arch Toxicol.* 2019 Feb;93(2):253-272.

Manuscript II

Schwartz, C. L., Vinggaard, A. M., Christiansen, S., Darde, T. A., Chalmel, F., and Svingen, T. Distinct transcriptional profiles of the female, male and finasteride-induced feminized male anogenital region in rat fetuses. *Tox Sci* 2019 169(1), 303–311

Manuscript III

Schwartz C. L., Svingen T., Christiansen S., Johansson, H. K. L., Pedersen M., Frandsen H. L., Frederiksen K. A., Govers L. C., Pask A. J. and Vinggaard A. M. Expression of estrogen and Wnt related factors in the rat perineal tissues during normal development and following disruption of fetal androgen action. *Manuscript in preparation*

Manuscript IV

Schwartz C. L., Svingen T. and Vinggaard A. M. Molecular markers of the sexually dimorphic perineum. *Study report*

Additional manuscripts within the area

Johansson H. K. L., **Schwartz C. L.**, Nielsen L. N., Boberg J., Vinggaard A.M., Bahl M.I. & Svingen T. Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, induces limited effects on adult rat testis. *Reprod Toxicol.* 2018 82:25-31

List of abbreviations

Abbreviation	Description
AGD	Anogenital distance
AGDi	Anogenital distance index
AMEN	Annotation, Mapping, Expression and Network
AMH	Anti-Müllerian hormone
ANOVA	Analysis of variance
AOP	Adverse outcome pathway
AC	Anus to clitoris
AF	Anus to fourchette
AP	Anus to anterior insertion of the penis
AR	Androgen receptor
AS	Anus to scrotum
BMI	Body mass index
BRB	Bulk RNA barcoding
DEG	Differentially expressed gene
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
EAS	Estrogen, androgen, steroidogenesis
EATS	Estrogen, androgen, thyroid, steroidogenesis
ED	Endocrine disruption
EDC	Endocrine disrupting chemical
ER	Estrogen receptor
Esr1	Estrogen receptor 1
EU	European Union
GD	Gestation day
GT	Genital tubercle
H&E	Hematoxylin & Eosin
Hh	Hedgehog
IC	Inhibitory concentration
IF	Immunofluorescence
INSL3	Insulin-like factor 3
IPCS	International Programme on Chemical Safety
KE	Key event

Abbreviation	Description
KER	Key event relationship
KO	Knock-out
LABC	Levator ani / bulbocarvernosus
LC-MS	liquid chromatography mass spectrometry
LH	Luteinizing hormone
MIE	Molecular initiating event
MPW	Masculinization programming window
NAM	New approach methodology
OECD	Organisation for Economic Co-operation and Development
Padi2	Protein-arginine deiminase type-2
PBTK	Physiologically based toxicokinetic
PND	Postnatal day
QIVIVE	Quantitative *in vitro* to *in vivo* extrapolation
QSAR	Quantitative structure-activity relationship
SEM	Standard error of the mean
Sfrp4	Secreted frizzled related protein 4
TDS	Testicular dysgenesis syndrome
US EPA	United States environmental protection agency
UV	Ultra violet
WHO	World Health Organization
Wnt	Wingless-like
Wnt2	Wingless-type MMTV integration site family member 2

Contents

1

Introduction

1.1 Rationale

Over the past decades, there has been an increase in male reproductive disorders such as hypospadias and cryptorchidism at birth, and low testosterone, poor semen quality and infertility in adulthood (Skakkebaek et al, 2016). Exposure to endocrine disrupting chemicals (EDCs) during development is believed to be a significant contributing factor to this trend in disease incidents (Diamanti-Kandarakis et al, 2009). Of particular concern for male reproduction are chemicals with anti-androgenic properties as they can perturb the masculinization process, especially if exposure occurs during critical stages of fetal development (van den Driesche et al, 2017; van den Driesche et al, 2012; Welsh et al, 2008; Welsh et al, 2007). Thus, to protect human reproductive health, much effort has been invested internationally in devising test strategies to screen chemicals for their endocrine disruptive potential.

One of the most reliable morphometric measurements we have to assess if a chemical exerts anti-androgenic effects in an intact organism is the anogenital distance (AGD) (Schwartz et al, 2019). The AGD is normally about twice as long in males as in females in humans and other mammals, and it is directly associated with fetal androgen levels (Hotchkiss & Vandenbergh, 2005; Salazar-Martinez et al, 2004). Consequently, a short male AGD is considered a marker for disrupted fetal androgen signaling and incomplete masculinization of the male fetus, and is associated with several male reproductive disorders (Dean & Sharpe, 2013; Skakkebaek et al, 2001; Thankamony et al, 2016). For this reason, measurement of AGD have within the last decade been incorporated into several OECD test guidelines used to test chemicals for reproductive toxicity (OECD, 2016a; OECD, 2016b; OECD, 2018a; OECD, 2018b). Despite the widespread use of AGD measurements, the exact molecular mechanisms that determine the AGD outcome remain poorly understood.

It is clear that blocking either steroidogenesis or androgen receptor (AR) action during fetal development can lead to short male AGD (Schwartz et al, 2019). However, chemicals with presumed estrogenic modes of action or with other, less defined, non-AR modes of action can also induce short male AGD (Boberg et al, 2016; Christiansen et al, 2014; Holm et al, 2015; Kristensen et al, 2011). To complicate matters further, some chemicals induce a longer rather than a shorter male AGD (Goetz et al, 2007; Manservisi et al, 2019; Taxvig et al, 2007). This suggests that there may be other regulatory pathways that play a role in determining AGD, at the very least as effect-outcome modifiers. A better mechanistic understanding of how chemicals affect AGD is therefore warranted. Such mechanistic knowledge could contribute to describing the detailed adverse outcome pathway (AOP) for short male AGD in the future.

The central premise for this PhD project was to investigate the signaling pathways involved in modulating AGD in rodents exposed to EDCs and to elucidate whether other signaling pathways, in addition to AR signaling, are involved in AGD development. Such knowledge may in the future provide a stepping-stone for the development of non-animal test methods in chemical hazard identification and risk assessment.

1.2 Hypothesis and aim

The aim of this PhD project was to identify signaling pathways affected in the perineal tissues of males with short AGD. The hypothesis was that the effects of EDCs on male AGD are not solely due to disruption of androgen signaling, but also includes other morphoregulatory pathways.

1.3 Methodology

To address the above stated hypothesis, four different approaches were applied. A simplified overview of the applied experimental methods is shown in Figure 1. First, I performed an extensive search of the current literature related to fetal exposure to xenobiotics and AGD measurements and used this to provide an overview of how different chemical classes affect AGD (chapter 3). Second, I carried out a transcriptomics study and RT-qPCR gene expression analyses on rat perineal tissues following *in utero* exposure to anti-androgenic compounds. This was done to identify signaling pathways that were affected in males with chemically induced short AGD (chapter 4 and chapter 5) (Figure 1A). Third, I set up ex vivo cultures of the perineum to investigate the transcriptional changes following 72h exposure to anti-androgenic compounds in culture medium (Figure 1B). Fourth, I investigated the temporal expression pattern of different genes in the perineal tissues to gain a better picture of the normal, sexually dimorphic development of the perineum (chapter 6) (Figure 1C).

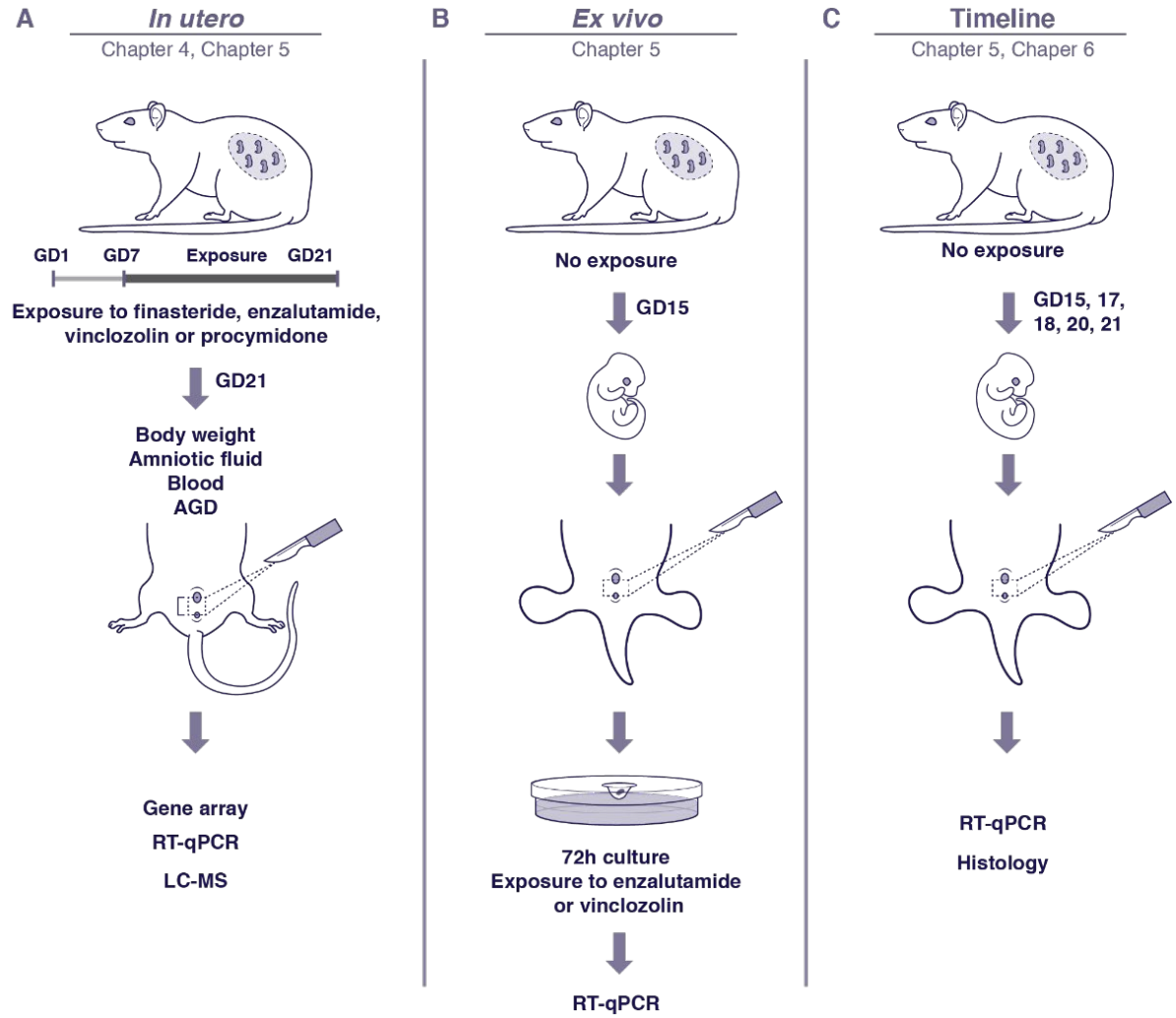

Figure 1. Simplified overview of the applied experimental approaches. A: Pregnant Sprague-Dawley rats were exposed by oral gavage to 10mg/kg bw/day finasteride (chapter 4), 10mg/kg bw/day enzalutamide (chapter 5), 40mg/kg bw/day procymidone (chapter 5) or 40mg/kg bw/day vinclozolin (chapter 5) from gestation day (GD)7-21. At GD21, fetuses were collected by cesarean section. Maternal and fetal parameters were recorded, maternal and fetal blood as well as amniotic fluid was collected, the anogenital distance (AGD) was measured and the perineum was isolated. Perineal tissues were analyzed by gene array analysis (chapter 4) and RT-qPCR (chapter 4 and chapter 5). Blood and amniotic fluid was analyzed by LC-MS (chapter 5). **B:** Fetuses were collected from non-dosed pregnant Sprague-Dawley rats at GD15 (chapter 5). Perineal tissues were isolated and grown in ex vivo culture where they were exposed to 1 or 100 µM of either enzalutamide or vinclozolin. After 72h in culture, the perineal tissues were collected for RT-qPCR analysis. **C:** Fetuses were collected from non-dosed pregnant Sprague-Dawley rats at GD15, -17, -18, -20 and -21 (chapter 5 and chapter 6). Perineal tissues were isolated and analyzed by RT-qPCR (chapter 5 and chapter 6) and histological analyses (chapter 6). Detailed descriptions of the applied materials and methods can be found in chapter 4-6.

1.4 Outline of the thesis

This chapter (chapter 1) has briefly introduced the area of research as well as the aim and methodologies central to this PhD project. Chapter 2 provides the theoretical background for the PhD thesis and introduces key concepts pertaining to endocrine disruption and concepts used in chemical risk assessment. It introduces the role of androgen in the process of sexual differentiation and how chemicals may affect this process. Lastly, it provides the current knowledge on perineal development and gives an introduction to the measurement of AGD. Chapter 3 presents an extensive literature review. The review introduces the fetal masculinization process and how it relates to the sexually dimorphic AGD. Central to this review is the comparison of how different chemical classes affect AGD. It provides a detailed discussion on the use of AGD measurements in a toxicological and clinical setting as a marker of fetal androgen action or risk of reproductive disorders. Chapter 4 presents a transcriptomics analysis of the perineum used to identify signaling pathways that are expressed in a sex-specific manner and are also affected in males with short AGD as a result of *in utero* exposure to the drug finasteride. Chapter 5 presents the temporal expression of the pathways identified in chapter 4 and investigates whether these pathways were also affected following *in utero* exposure to compounds with AR antagonistic modes of action. In addition, chapter 5 provides estimates of the *in vivo* anti-androgenic potency of four different compounds to assess if this can be used to predict AGD outcome. Chapter 6 presents a preliminary study investigating the temporal expression of genes that, according to previous literature, play a key role in the sexually dimorphic development of the perineum. In chapter 7, the results presented in chapter 3-6 are discussed in a broader context. Lastly, possible future perspectives as well as conclusions on the thesis are provided.

1.5 References

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2

Background

2.1 Endocrine disruption

2.1.1 Endocrine disrupting chemicals

Thousands of industrial chemicals are manufactured each year, often in staggering volumes, and are used in most of our everyday products such as food and food packaging materials, cosmetics, clothes, furniture, electronic devises and building materials. From here, they may enter the body by ingestion, inhalation or skin absorption. Some of these chemicals can interfere with the human endocrine system and cause adverse health effects in the exposed individual or its offspring. The World Health Organization (WHO) define such endocrine disrupting chemicals (EDCs) as "*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*" (IPCS, 2002). Exposure to EDCs during fetal life is of particular concern, as EDCs can disrupt the tightly regulated endocrine signaling that direct many aspects of fetal development. This includes the development of the reproductive system that, to a large extent, is governed by endocrine action. Early life exposure to EDCs such as phthalates, bisphenols and mild analgesics may be associated with adverse effects on human reproductive health in both males and females (Fisher et al, 2016; Johansson et al, 2017; Lind et al, 2017; Mammadov et al, 2018; Skakkebaek et al, 2001; Swan et al, 2005).

The Organization for Economic Co-operation and Development (OECD) has established test guidelines as tools for assessing the potential effects of chemicals on human health and the environment. They are accepted internationally as standard methods for safety testing of chemicals and are used to assess the reproductive toxicity of chemicals and, in some cases, their endocrine disruptive (ED) potential. These guidelines include both *in vitro* assays assessing for example induction or inhibition of nuclear steroid receptor or steroid synthesis (OECD, 2011; OECD, 2016c; OECD, 2016d) and *in vivo* assays using *in utero* exposure to asses reproductive and

> The Organization for Economic Co-operation and Development (OECD) is an intergovernmental organization with representatives from 30 industrialized countries in North America, Europe, Asia, and Pacific region. The OECD has established test guidelines for the testing of chemicals to be used by government, industry and independent laboratories to identify and characterize potential hazards of chemicals. Experts and scientists from research and regulatory areas as well as industry, non-governmental organizations, and animal protection organizations help to ensure sound science and international regulatory acceptance of test methods.
> (OECD.org)

ED related endpoints in the offspring (OECD, 2016a; OECD, 2016b; OECD, 2018b; OECD, 2018c).

2.1.2 Anogenital distance

The anogenital distance (AGD) is the distance from the anus to the external genitalia and it is approximately twice as long in males as in females in both rodents, and humans (Hotchkiss & Vandenbergh, 2005; Salazar-Martinez et al, 2004). AGD is directly related to androgen signaling and measurement of AGD can therefore be used to retrospectively determine androgen action during fetal life. As a result, short male AGD is considered a marker of incomplete masculinization. Several rodent studies show that exposure of the developing male fetus to EDCs via the mother (*in utero*) induces short AGD in the male offspring (see chapter 3). A short male AGD in rodents is associated with reproductive disorders such as hypospadias and cryptorchidism at birth, as well as decreased testosterone levels and reduced semen quality later in life (see chapter 3 for details, (Christiansen et al, 2008; Zhang et al, 2014)) (Figure 1). In humans, short male AGD is associated with hypospadias and cryptorchidism at birth (Hsieh et al, 2008; Jain & Singal, 2013; Thankamony et al, 2014), as well as reduced semen quality, decreased serum testosterone, impaired fertility and testicular germ cell tumors in adulthood (Eisenberg et al, 2011; Eisenberg et al, 2012; Mendiola et al, 2011; Moreno-Mendoza et al, 2020) (Figure 1). In addition, some epidemiological studies also report an association between fetal exposure to EDCs such as phthalates, dioxins, bisphenols, and mild analgesics and short male AGD (see chapter 3 (Fisher et al, 2016; Lind et al, 2017; Mammadov et al, 2018; Swan et al, 2005; Vafeiadi et al, 2013). Albeit, a direct cause-effect relationship is difficult to prove.

Based on the similarities between rodents and humans as outlined, the effects on AGD in toxicological animal studies are considered relevant for human reproductive health. AGD is therefore included as a mandatory ED sensitive endpoint in several OECD test guidelines for reproductive toxicity (OECD, 2016a; OECD, 2016b; OECD, 2018a; OECD, 2018b; OECD, 2018c). Detailed descriptions of AGD, its measurement and use, are found in section 2.5-*Measurement of AGD* and in chapter 3.

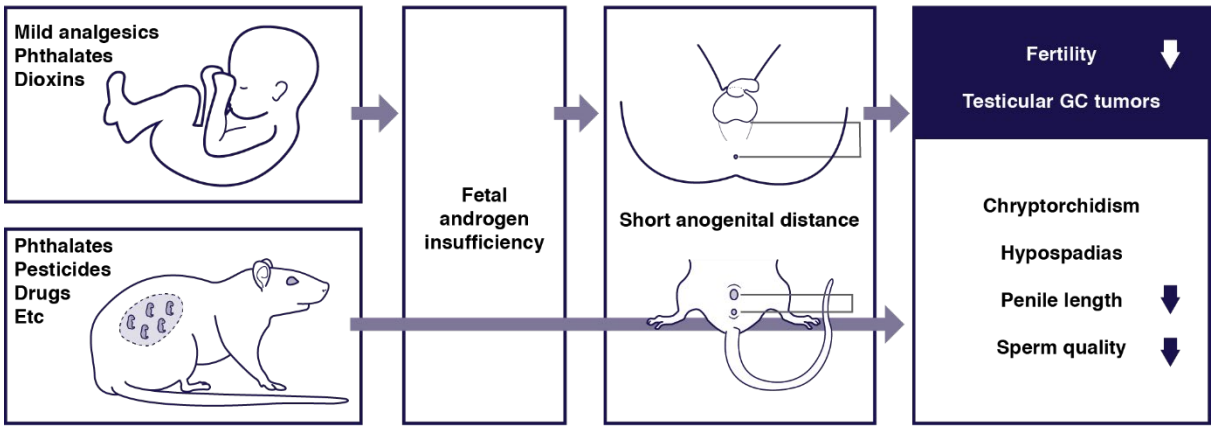

Figure 1. Anogenital distance as a biomarker for incomplete masculinization. Exposure to EDCs during fetal life can disrupt androgen signaling and induce a short AGD in males. Short male AGD is associated with disorders such as cryptorchidism, hypospadias, decreased penile length and reduced sperm quality in both rodents and humans. In addition, short male AGD is associated with reduced fertility and testicular germ cell (GC) tumors in humans. Short male AGD is therefore used as a retrospective marker of fetal androgen activity and a predictor of future reproductive health. In humans, a direct cause-effect relationship is difficult to prove as indicated in the figure by the three arrows at the top. The idea for this figure was adopted from (Svingen et al, 2017).

2.1.3 Adverse outcome pathways and new approach methodologies

Chemical hazard identification and risk assessment has for many years relied heavily on data obtained from animal studies. With the immense numbers of industrial chemicals that need testing, however, the use of animal studies raises issues concerning both animal welfare, high cost and low throughput. As a result, there is an increasing focus on alternative, non-animal test methods (Parish et al, 2020). In 2019, the administrator of the United States Environmental Protection Agency (US EPA), Andrew Wheeler, signed a directive that calls for the agency to:

"*Reduce its requests for, and funding of, mammal studies by 30 percent by 2025, and eliminate all mammal study requests and funding by 2035*" (epa.gov)

This phasing out of animal studies highlights the imminent need for representative, new approach methodologies (NAMs) for the continuous protection of human health. To predict the effects of chemicals on reproductive health, much effort is put into developing NAMs to test for reproductive toxicity and endocrine disruption. These approaches include high

throughput *in vitro* assays, physiologically based toxicokinetic (PBTK) models and quantitative structure-activity relationship (QSAR) models developed to predict estrogen, androgen and steroidogenesis (EAS) activity (Judson et al, 2018). Data from such NAMs have been deemed robust enough to be included, together with data from animal studies, in the weight-of-evidence assessment of chemicals in a regulatory setting (Judson et al, 2018).

The current NAMs often focus on the classic estrogen, androgen, thyroid, and steroidogenic (EATS) modalities for endocrine disruption. For reproductive toxicology, this often means the estrogen, androgen, and steroidogenic (EAS) modalities. However, as highlighted in a Detailed Review Paper by the OECD back in 2012, non-EATS modalities may also contribute to ED effects (OECD, 2012). That is, chemicals may induce adverse effects by mechanisms not classically investigated in chemical risk assessment. The OECD highlights seven non-EATS modalities, namely hypothalamic–pituitary–gonadal, hypothalamic–pituitary–adrenal, somatotropic, retinoid, hypothalamic–pituitary–thyroid, vitamin D and peroxisome proliferator-activated receptor (PPAR). These are signaling pathways for which there is "(a) significant evidence of susceptibility to disruption by environmental chemicals with potential for adverse outcome exists; and (b) assay procedures for the detection of environmental endocrine disruption that are sufficiently developed for protocol standardization and validation" (OECD, 2012). Other pathways for which these two requirements cannot be met (yet), may also contribute to the non-EATS modalities for ED. Examples could be the evolutionarily conserved Wnt and hedgehog (HH) pathways as recently reviewed elsewhere (Johansson & Svingen, 2020).

The existence of non-EATS modalities has important implications if we are to rely solely on NAMs for predicting the reproductive toxicity of chemicals. Increased reliance on NAMs requires detailed mechanistic knowledge of the toxicological pathways to accurately mimic effect outcomes in an intact organism. If we for example only use NAMs based on the classical nuclear receptor assays and steroidogenesis assays we may wrongly classify a chemical as "safe" because the relevant modality is not tested. By contrast, chemicals acting by non-EATS modalities would be detected in intact animal studies because they are not limited to a set number of modalities. This does not mean that non-EATS modalities are not already included when testing chemicals for potential ED effects; rather, we do not know if the ones included are sufficient to cover all relevant modalities. Thus, to ensure reliable chemical risk assessment in a non-animal testing era, we need mechanistic insight to assess if the existing NAMs sufficiently mimic the toxicological pathways *in vivo* or whether they should be refined or new ones developed.

An emerging tool in chemical risk assessment, and in the development and refinement of NAMs, is the adverse outcome pathway (AOP) framework. The aim of the AOP framework, now run by the OECD, is to integrate knowledge from molecular and developmental biology, as well as *in vitro* and *in vivo* toxicology, to describe the mechanistic events leading from the initial molecular trigger to the adverse outcome at the level of the organism (Ankley et al, 2010; Ankley & Edwards, 2018; Draskau et al, 2020). A detailed description of the AOP concept can be found elsewhere (Ankley & Edwards, 2018; OECD, 2018d; Villeneuve et al, 2014). Briefly, an AOP consists of modular components that describe the molecular initiating event (MIE) triggering a series of key events (KEs) that are causally linked by key event relationships (KERs) ultimately leading to an adverse outcome (AO). Individual AOPs can be compiled into AOP networks linked by shared KEs. Important to the AOP principle is that AOPs are simplifications of complex biological processes. Rather than covering all molecular and cellular events that take place in the organism, AOPs highlight the key events that play a significant role in the progression towards an adverse outcome. The different modules are not chemical-specific so knowledge from one AOP can be used to develop new AOPs even if only a few of the events are common to the given pathways. Lastly, to ensure usefulness in the regulatory setting, the different KEs must be testable (or measurable), highlighting how AOPs can be used in the development of new NAMs.

One example of an AOP network consists of three putative AOPs pertaining to short male AGD (Figure 2). These three AOPs (AOPwiki, 305; AOPwiki, 306; AOPwiki, 307) originate from three individual MIEs: inhibited steroidogenesis, 5α-reductase inhibition and androgen receptor (AR) antagonism. They have common KEs such as decreased AR mediated gene transcription and altered perineal development and they ultimately lead to short male AGD, which, in a regulatory context, is considered an adverse outcome. If new MIEs or KEs for short male AGD are identified, they can be incorporated into the existing AOPs or be used to develop new ones as discussed in chapter 7.

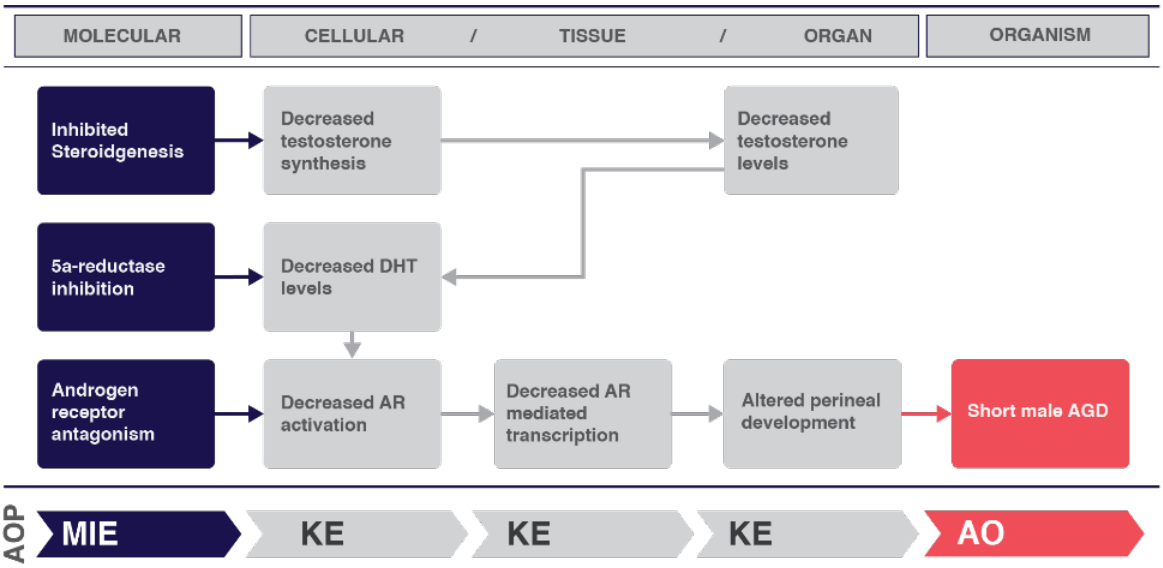

Figure 2. Possible adverse outcome pathway (AOP) network for short male anogenital distance (AGD). Based on the current mechanistic knowledge, three AOPs for short male AGD are under development at AOPwiki.org (AOPwiki, 305; AOPwiki, 306; AOPwiki, 307). There are three individual molecular initiating events (MIEs; dark blue boxes). The MIEs lead to different key events (KEs; grey boxes) at either the cell, tissue or organ level. The KEs are connected by causally linked key event relationships. Ultimately, the KEs lead to the adverse outcome (AO; red box) at the organism level, which in this case is short AGD. The three AOPs share several KEs and can therefore be compiled into an AOP network.

2.2 Sexual differentiation

2.2.1 Sex determination and masculinization

In mammals, the XY ('male') and XX ('female') fetuses are initially indistinguishable from each other. Differentiation of the bipotential gonad into either testis or ovaries is genetically controlled by expression of the sex-determining gene *Sry* encoded by the Y-chromosome (Koopman et al, 1991; Sinclair et al, 1990). Expression of *Sry* in the XY gonads initiates a molecular chain of events leading to testis development (Svingen & Koopman, 2013). In the absence of *Sry* expression, as in the XX gonads, opposing regulatory pathways will instead direct ovarian differentiation (Nicol & Yao, 2014). Expression of *Sry* triggers the differentiation of pre-Sertoli cells, which orchestrate the organization of testis cords. In the surrounding interstitial space, the fetal Leydig cells are formed and will become the primary site for androgen production (Svingen & Koopman, 2013). The testes also produce insulin-like factor 3 (INSL3), that induce transabdominal descent of the testes (Nef & Parada, 1999) and anti-Müllerian hormone (AMH) that induce regression of the anlage for the female

reproductive tract called the Müllerian ducts (Josso et al, 1993). Androgens drive the development of the secondary male sex organs and the general masculinization of the fetus (Jost, 1947; Jost, 1953; Macleod et al, 2010). Masculinization of the fetus takes place during a short time span of fetal development referred to as the masculinization programming window (MPW) (van den Driesche et al, 2017; van den Driesche et al, 2012; Welsh et al, 2008). In rats, the MPW ranges from embryonic day (e)15.5 to e18.5 corresponding approximately to gestation week 8 to 14 in humans. Androgen action during the MPW is believed to program all the male reproductive organs, thus determining their ultimate adult size and function (Macleod et al, 2010; Welsh et al, 2008). This is important for the implications of exposure to EDCs during fetal life as described further below.

2.2.2 Androgen synthesis

Testosterone is produced in the fetal Leydig cells by the process of steroidogenesis in a stepwise conversion of cholesterol (Figure 3) (Scott et al, 2009). Here, cholesterol is transported into the mitochondrial matrix by StAR and is converted to pregnenolone by CYP11A. Pregnenolone translocates from the mitochondria to the smooth endoplasmic reticulum where the conversion into androgens take place by the enzymatic action of 3β-HSD, CYP17 and 17βHSD (Scott et al, 2009). Testosterone can then be secreted to the circulation where it is bound to specific binding proteins. At androgen target tissues, unbound testosterone can diffuse into the target cell and bind the androgen receptor (AR). In certain target tissues, testosterone is converted to the more potent dihydrotestosterone (DHT) by the enzyme 5α-reductase (Scott et al, 2009). DHT drives the masculinization of tissues such as the external genitalia and prostate (Azzouni et al, 2012; Imperato-McGinley et al, 1992) and induces regression of the nipple anlagen in male rodents (Imperato-McGinley et al, 1986). DHT is also responsible for sex-specific differentiation and growth of the perineum and therefore a determining factor of AGD (Bowman et al, 2003; Christiansen et al, 2009). In humans, DHT can also be produced by a so-called "backdoor pathway" where placental progesterone is converted to androsterone in the placenta, liver, adrenals and, to a lesser degree, the fetal testis. In this case, DHT is produced at the target tissues by the enzymatic action of *ANKR1C2* and *ANKR1C4* (Fluck et al, 2011; O'Shaughnessy et al, 2019).

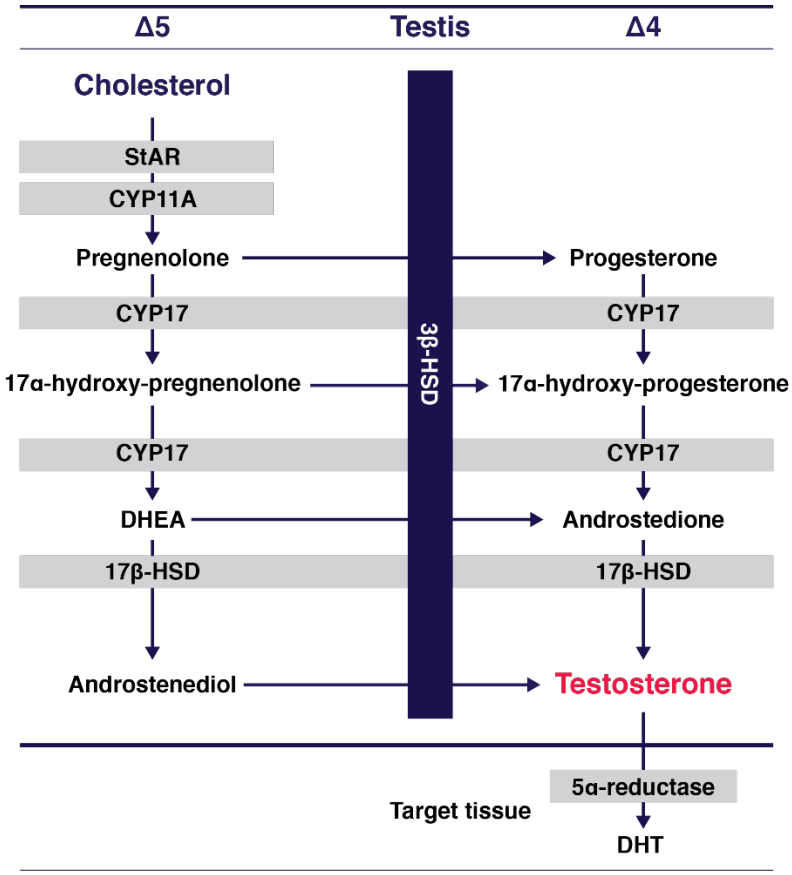

Figure 3. Steroidogenesis in the fetal testis. Cholesterol is converted to pregnenolone by the joint action of StAR and CYP11A. Pregnenolone is then converted to testosterone by enzymatic action of 3β-HSD, CYP17 and 17βHSD. In the Δ5 pathway, the intermediates are 17α-hydroxy-pregnenolone, DHEA, and androstenediol. In the Δ4 pathway, the intermediates are progesterone, 17α-hydroxy-progesterone, and androstenedione. Testosterone is converted to DHT at the target tissues by the enzyme 5α-reductase.

2.2.3 Androgen receptor signaling

Testosterone or DHT binds the AR in the cytoplasm of the target cell. AR is a nuclear hormone receptor and functions as a homodimer (Figure 4). Upon ligand binding, the androgen-AR complex translocates to the nucleus where it binds to specific hormone response elements on the DNA, thereby regulating transcription of AR target genes (Roy et al, 1999). In addition, some effects of androgens may be mediated by non-genomic actions by induction of second messenger signal transduction cascades such as activation of MAPK or increases in free intracellular calcium (Heinlein & Chang, 2002).

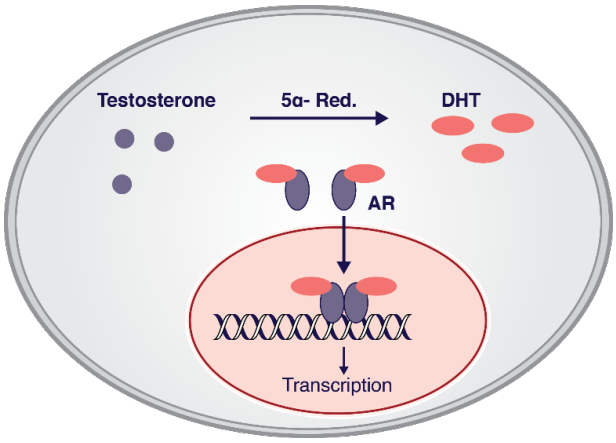

Figure 4. Androgen receptor (AR) signaling. At the target tissue, testosterone diffuses into the cells and is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT binds the AR with high affinity. AR dimerizes and translocates to the nucleus where it binds specific androgen response elements on the DNA to regulate transcription of AR target genes. Testosterone itself can also bind the AR to regulate transcription, albeit with a lower affinity than DHT.

2.2.4 Mechanisms of endocrine disruption

Endocrine disruption can occur through interruption with any of the processes outlined above. EDCs can affect hormone levels by disrupting synthesis, bioavailability or metabolism of endogenous hormones. In addition, EDCs can interfere with signaling of the endogenous hormones by acting as either receptor agonist or antagonist, thereby inducing or inhibiting receptor activity. As mentioned previously, EDCs may also act by non-EATS modalities. Currently, there is evidence to support three mechanisms by which EDCs can induce short male AGD (Figure 2 and Figure 5). This is by inhibiting steroidogenesis, inhibiting 5α-reductase activity or antagonizing AR. However, chemicals believed to have a mainly estrogenic effect such as butyl paraben and bisphenol A or non-AR-mediated mode of action such as paracetamol have also been found to affect AGD in some studies (Boberg et al, 2016; Christiansen et al, 2014; Holm et al, 2015; Kristensen et al, 2011; Zhang et al, 2014), suggesting that other modalities might be involved.

2.3 Anti-androgenic chemicals

Compounds with anti-androgenic activity cover many different chemical classes such as plasticizers, pharmaceuticals, pesticides, UV-filters and preservatives. The animal studies conducted in this PhD project included *in utero* exposure to both pharmaceuticals and pesticides. The chosen compounds had different anti-androgenic mechanisms of action,

different potency and selectivity and had previously been shown to induce short male AGD as described below. As such, they were used as model compounds to study the effects in the perineum of males with short AGD induced by different anti-androgenic mechanisms. Exposure to the used pharmaceuticals is not considered a concern in real life, as they are used to treat adult men for prostate cancer. They serve their purpose in this experimental setting where we are specifically looking at uncovering affected signaling pathways in the perineal tissues. A short introduction to each of the included compounds is given below and shown in Figure 5.

2.3.1 Finasteride

Finasteride is a synthetic anti-androgen used in humans to treat hair loss, benign prostate hyperplasia and, at high doses, prostate cancer (Clark et al, 1990; FDA, 2011; Imperato-McGinley et al, 1992). Finasteride inhibits the 5α-reductase enzyme and thereby the peripheral conversion of testosterone to DHT (FDA, 2011). Studies of *in utero* exposure to finasteride report male offspring with increased incidence of hypospadias, reduced prostate weight, nipple retention, and short male AGD (Bowman et al, 2003; Christiansen et al, 2009; Clark et al, 1993).

2.3.2 Enzalutamide

Enzalutamide is a synthetic, second generation, antiandrogen used in humans to treat metastatic castration-resistant prostate cancer (Shore et al, 2016). Enzalutamide is an AR antagonist and targets three key stages of AR signaling: blocking androgen binding, inhibiting translocation of activated AR and inhibiting binding of activated AR to the DNA (Tran et al, 2009). In contrast to first generation AR antagonists like flutamide, enzalutamide has no AR agonistic effects at high doses (Tran et al, 2009). Enzalutamide has not previously been used in studies of reproductive toxicity, but one previous report reports short male AGD in mice following *in utero* exposure to the compound (TGA, 2014).

2.3.3 Vinclozolin

Vinclozolin is a dicarboximide fungicide used to control diseases such as blights, mold and rot in for instance vineyards. Vinclozolin is not approved for use in the EU (EC, 2007). Vinclozolin is quickly metabolized *in vivo* to the metabolites M1 and M2 both of which have anti-androgenic activity (Kelce et al, 1994). Both vinclozolin and its metabolites show AR antagonistic activity *in vitro* (Kelce et al, 1994; Nellemann et al, 2003; Scholze et al, 2020; Vinggaard et al, 2008). Toxicological studies in rats show that *in utero* exposure to vinclozolin results in several adverse effects on the male offspring including hypospadias, cleft

phallus, cryptorchidism, reduced prostate weight, vaginal pouch, nipple retention, and female-like short male AGD at high doses (Christiansen et al, 2009; Gray et al, 1994; Hass et al, 2007; Matsuura et al, 2005; Wolf et al, 2004).

2.3.4 Procymidone

Procymidone is also a dicarboximide fungicide and, like vinclozolin, its use is not permitted in the EU (EC, 2008). Procymidone also shows AR antagonistic activity *in vitro* (Nellemann et al, 2003; Scholze et al, 2020) and have adverse effects on male offspring following *in utero* exposure such as hypospadias, cryptorchidism, reduced prostate and weight, nipple retention, and short male AGD (Hass et al, 2012; Hass et al, 2007; Wolf et al, 1999).

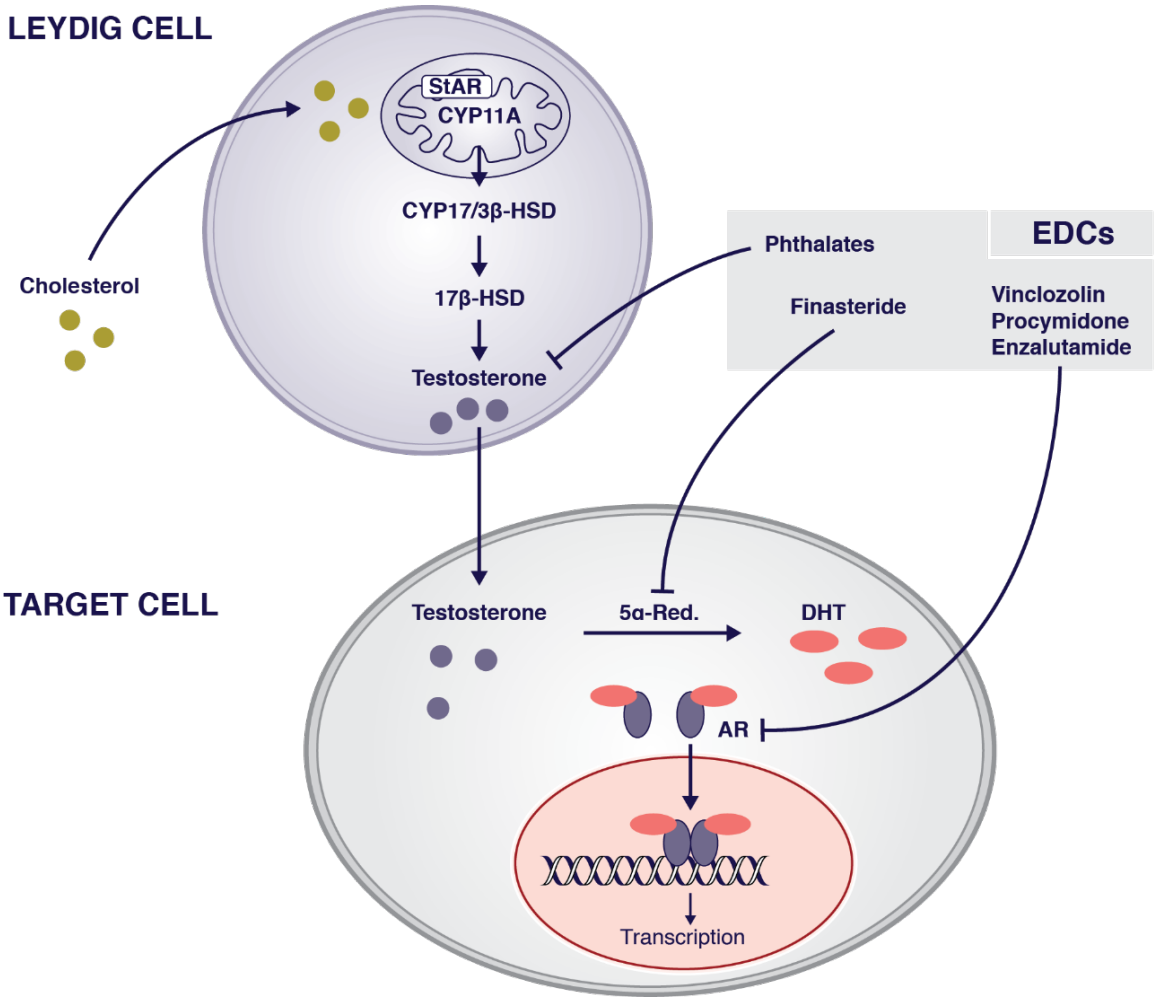

Figure 5. Mechanisms for endocrine disruption by endocrine disrupting chemicals (EDCs) with an anti-androgenic mode of action. Compounds such as the synthetic anti-androgen finasteride inhibit the enzyme 5α-reductase thereby blocking the conversion of testosterone to DHT. Other compounds such as the synthetic anti-androgen enzalutamide and the two fungicides vinclozolin and procymidone act as AR antagonists. Other EDCs, such as phthalates, reduce testosterone synthesis. All of these three main mechanisms ultimately result in decreased AR activity and thus incomplete masculinization.

2.4 Perineal development

Striated muscle is one of the non-reproductive tissues that are masculinized by androgens. The levator ani/bulbocavernosus (LABC) muscles of the perineum are especially sensitive to androgen action as evidenced by the significant weight reduction in LABC following AR ablation in rats (Chambon et al, 2010; MacLean et al, 2008; Ophoff et al, 2009). Consequently, females have only remnant LABC at birth, an effect that can be reversed by treatment of testosterone propionate during gestation (Cihak et al, 1970; Tobin & Joubert,

1988). In males, LABC attach to the base of the phallus and is implicated in erection and ejaculation (Karacan et al, 1983; Sachs, 1982). The size of LABC is thought to directly affect AGD as ablation of AR in mice reduces LABC weight and induce a female-like, short male AGD (Ipulan et al, 2014). In agreement, several toxicological studies in rodents report reduced LABC weight and short male AGD following *in utero* exposure to anti-androgens (Bowman et al, 2003; Christiansen et al, 2009; McIntyre et al, 2001; Wolf et al, 1999). Although the anabolic effects of androgens on skeletal muscle have been known for decades the molecular basis for its effects remain poorly understood. It is possible that fetal androgen action directs the sexually dimorphic growth and development of LABC by regulation of cell death and cell proliferation. Ablation of the pro-apoptotic genes *Bax* and *Bak* in mice result in a great increase in the size of LABC in young adult females, albeit it is not completely masculinized (Jacob et al, 2008). In addition, ablation of AR significantly reduced the percentage of proliferating cells in the BC of male mice at e15.5 (Ipulan et al, 2014).

In addition to muscle cells, skeletal muscle is composed of several cell types, many of which express AR including satellite cells, fibroblasts, vascular endothelial cells, nerve cells and mesenchymal cells (Johansen et al, 2007; Monks et al, 2004; Sinha-Hikim et al, 2004). Cell type-specific knock out (KO) studies of AR in mice show conflicting results. While some studies show decreased size of the LABC following muscle specific KO (Chambon et al, 2010; Ophoff et al, 2009) another find that ablation of AR in non-myocytic cells, but not in muscle cells, affect LABC size (Ipulan et al, 2014). In the latter study, immunofluorescence staining shows that non-myocytic cells, and not myocytic cells, are AR positive.

A better mechanistic insight into how androgens mediate the sexually dimorphic development of the LABC could add to the knowledge of how EDCs might affect AGD and could contribute to expanding on the existing AOPs for short male AGD. This is the focus of chapter 4, 5 and 6 and is discussed separately in each of these chapters and collectively in chapter 7.

2.5 Measurement of AGD

In both humans and rodents, measurement of AGD at birth can be used to retrospectively assess fetal androgen action. In rodents, AGD has been used for sex determination of newborns for a long time. In newborn rats, the genital tubercle is present in both sexes and the males have no scrotum and undeveloped external genitalia. AGD in rats is therefore measured from the anus to the insertion of the genital tubercle (Figure 6A). There is significant correlation between AGD and body weight in rats (Gallavan et al, 1999) and body weight should therefore be taken into account in the assessment of AGD. Using the AGD/body weight ratio is, however, not optimal as body weight can be regarded as a three

dimensional factor which should be taken into account. Therefore, calculating the anogenital index (AGDi) is recommended in rodent studies, which is done by dividing AGD with the cube root of body weight. Body weight should also be included as a covariate in the statistical analysis of AGD (Gallavan et al, 1999; OECD, 2008; OECD, 2018a)

In humans, the measurement of AGD is relatively new (Salazar-Martinez et al, 2004), but is increasing in both epidemiological and clinical contexts. A limitation to the use of human AGD data has been the lack of reference values. An important contribution in this regard comes from the International AGD consortium (IAC) that recently presented age-, sex-, and method-related reference levels for AGD based on measurements of more than 3500 healthy infants (Fischer et al, 2020). There are two main ways of measuring AGD in humans (Figure 6B). The shorter of the two measurements is from the anus to the junction between the perineum and the scrotum (AGD_{AS}) in boys and from the anus to the fourchette (AGD_{AF}) in girls. The longer measurement is made from the anus to the anterior insertion of the penis (AGD_{AP}) in boys and from the anus to the clitoris (AGD_{AC}) in girls. Adjustments for body weight of the infant can be done for example by calculating the AGD/body mass index (BMI; kg/m^2) ratio (Fischer et al, 2020).

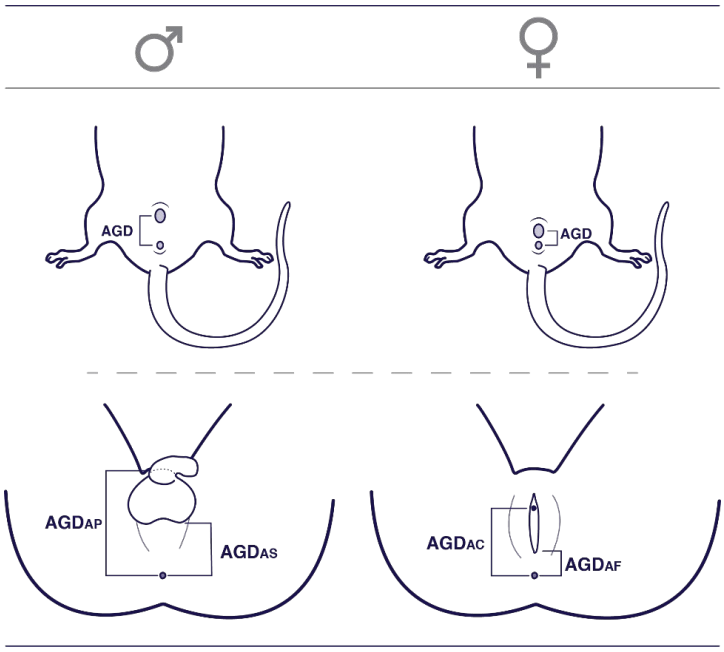

Figure 6. Measurement of anogenital distance (AGD). A: In rodents, where the genital tubercle is present in both males and females at birth and the males have no scrotum and undeveloped external genitalia, the AGD is measured from the anus to the insertion of the genital tubercle. **B:** In humans, there are two main ways of measuring AGD. The short measurement is from the anus to the junction between the perineum and the scrotum (AGD_{AS}) in boys and from the anus to the fourchette (AGD_{AF}) in girls. The long measurement is from the anus to the anterior insertion of the penis (AGD_{AP}) in boys and from the anus to the clitoris (AGD_{AC}) in girls.

The measurement of AGD is, as previously mentioned, mandatory to perform in several OECD test guidelines (OECD, 2016a; OECD, 2016b; OECD, 2018b; OECD, 2018c) where it is used as a sensitive endpoint for ED in a regulatory setting. *In utero* exposure to many different chemical compounds have been shown to induce short male AGD in rodent studies. In addition, human epidemiological studies have found associations between fetal exposure to EDCs and effects on AGD. In the next chapter (chapter 3) the available literature pertaining to *in utero* xenobiotic exposure and AGD measurements in rodents and humans is presented and discussed.

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Manuscript I

3.1 Manuscript I

Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders

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REVIEW ARTICLE

Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders

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Abstract

Male reproductive development is intricately dependent on fetal androgen action. Consequently, disrupted androgen action during fetal life can interfere with the development of the reproductive system resulting in adverse effects on reproductive function later in life. One biomarker used to evaluate fetal androgen action is the anogenital distance (AGD), the distance between the anus and the external genitalia. A short male AGD is strongly associated with genital malformations at birth and reproductive disorders in adulthood. AGD is therefore used as an effect readout in rodent toxicity studies aimed at testing compounds for endocrine activity and anti-androgenic properties, and in human epidemiological studies to correlate fetal exposure to endocrine disrupting chemicals to feminization of new-born boys. In this review, we have synthesized current data related to intrauterine exposure to xenobiotics and AGD measurements. We discuss the utility of AGD as a retrospective marker of in utero anti-androgenicity and as a predictive marker for male reproductive disorders, both with respect to human health and rodent toxicity studies. Finally, we highlight four areas that need addressing to fully evaluate AGD as a biomarker in both a regulatory and clinical setting.

Keywords Anogenital distance · Reproduction · Endocrine disruptors · Toxicology · Risks assessment

Introduction

Few things permeate our lives more than our sex and reproductive capacity, influencing not only our physical characteristics, but also behaviour and social perception. Ensuring proper reproductive development and life-long health therefore seems obvious, yet modern living is increasingly putting pressure on the processes of sexual differentiation and reproductive function. Exposure to endocrine disrupting chemicals (EDCs) during fetal life in both males and females has been raised as particularly disconcerting, since this is the period when the sexual organs form and, in many respects, lay the foundation for adult reproductive health (Johansson et al. 2017; Skakkebaek et al. 2016). Much effort has thus been invested in understanding the relationships between fetal exposure to xenobiotics and reproductive disorders, as well as to devise testing strategies to screen chemicals for potential endocrine disrupting effects.

In males, reproductive disorders associated with impaired fetal testis development or function vary in both phenotype and time of manifestation. Often described by the 'testicular dysgenesis syndrome' hypothesis (Skakkebaek et al. 2016), these male disorders range from hypospadias and cryptorchidism in infants (Hsieh et al. 2008, 2012; Jain and Singal 2013; Thankamony et al. 2014), to low testosterone levels, impaired semen quality and fertility issues in adult men (Eisenberg et al. 2011, 2012; Mendiola et al. 2011). Because of this complexity, it is difficult to adopt a single biomarker to use in animal toxicity studies aimed at testing chemicals for potential adverse effects on male reproductive health. The anogenital distance (AGD), however, is considered a broad biomarker capable of both retrospectively determine

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early-life androgen disruption and predict late-life reproductive disorders in male offspring (Dean and Sharpe 2013; Thankamony et al. 2016).

The AGD refers to the distance between the anus and the external genitalia, and is approximately twice the length in male compared to female new-borns. This sexual dimorphism is apparent in rodents as well as humans (Salazar-Martinez et al. 2004; Thankamony et al. 2009) and is a consequence of the androgen-driven dimorphic differentiation of the two sexes (Fig. 1). This sexual bifurcation extends to the perineum, where a muscular complex develops in males, but not in females. A short male AGD is, therefore, considered a marker of disrupted androgen action. In rodents, a short male AGD largely predicts the same adverse effect outcomes as in humans. In fact, the idea of investigating AGD in human epidemiological studies came from rodent developmental and reproductive toxicity studies, where AGD had been used for decades as a marker of impaired fetal androgen action and regarded as a an adverse outcome (Tables 1, 2).

In this review, we discuss current knowledge concerning AGD measurements, both as a clinical marker in humans and as a morphometric measure of fetal androgen disturbance in rodent toxicity studies. We have collated a growing body of toxicological studies that have reported on AGD measurements to gain a better overview of what evidence support the exclusive androgen-driven masculinization thesis, or to tease out other potential mechanisms that may lead to similar effects on AGD. However, first, to better appreciate why the AGD is a useful marker to assess early-life androgen disruption, we need to broadly outline how the two sexes develop.

Sexual differentiation and the importance of early androgen signaling

From gonadal sex determination to testosterone synthesis

At first, the male and female embryos are morphologically indistinguishable and only differentiate down two distinct trajectories after the appearance of either testes or ovaries. The specification of reproductive sex, or gonadal sex determination, is genetically controlled by the Y-linked sex determining gene *Sry* that is expressed in XY, but not in XX gonads, triggering testis differentiation in male fetuses (Koopman et al. 1991; Svingen and Koopman 2013). Subsequently, the testes differentiate into compartmentalized organs comprising testis cords and an interstitial space. Fetal Leydig cells differentiate within the interstitium and become the main site of androgen synthesis necessary for development of accessory male sex organs and general masculinization of the body (Svingen and Koopman 2013). The testes also produce the peptide hormone Insulin-like factor 3 (INSL3) that is required for transabdominal testicular descent (Nef and Parada 1999), and anti-Müllerian hormone (AMH) which ensures regression of the Müllerian ducts; a paired structure that otherwise would develop into the female reproductive tract (Behringer 1994; Josso et al. 1993). In simple terms, the absence of these 'male-centric' factors allows for the female reproductive system to develop.

Testosterone drives masculinization

Simply put, testosterone regulates secondary sex differentiation. If testosterone is present, the body will develop male traits; if not present, the body will develop female traits (Fig. 1). However, the full picture is much more complex. First, many factors other than testosterone are involved, including AMH and INSL3 as mentioned above, but also others such as Hedgehog (Hh), Wingless-like (Wnt) and various growth factors. Second, androgen-dependent masculinization processes are likely influenced by actual hormone levels in target tissues, meaning that masculinization cannot be viewed as a simple 'on–off' switch, but rather a scenario, where more or less androgens can, to some degree, result in more or less masculine traits. From this viewpoint, it is more difficult to define when a morphometric change can be regarded as adverse or not. Nevertheless, as shown by Alfred Jost more than half a century ago (Jost 1947, 1953), androgens are the main drivers of male sex differentiation and are essential during a critical time window of development (MacLeod et al. 2010).

The fetal masculinization process involves numerous tissues and organs, including development of external genitalia and sex-specific differentiation of the perineum: the region between anus and genitalia (Fig. 1). In these tissues, the process seems largely governed by dihydro-testosterone (DHT) which is locally converted from testosterone by the enzyme 5α-reductase. DHT acts by binding to and activating the Androgen receptor (AR), a nuclear hormone receptor responsible for regulating transcription of target genes (Fig. 2).

It has been suggested that the AGD is masculinized during development when AR activation stimulates the growth of the perineal muscles *levator ani* and *bulbocavernosus* (LABC) complex. In males, AR is expressed in non-myocytic cells of the LABC (Ipulan et al. 2014). Activation of AR in these cells prompts the growth of the LABC complex and the resulting size is thought to directly affect the AGD. Indeed, ablation of AR in rodents impairs development of the LABC complex and results in feminized male AGD (Ipulan et al. 2014; MacLean et al. 2008; Notini et al. 2005).

It is likely that more subtle changes in androgen levels, as for instance seen with fetal exposure to anti-androgenic compounds, can affect AGD by the same mechanism. This is supported by rodent toxicity studies, where a short AGD is

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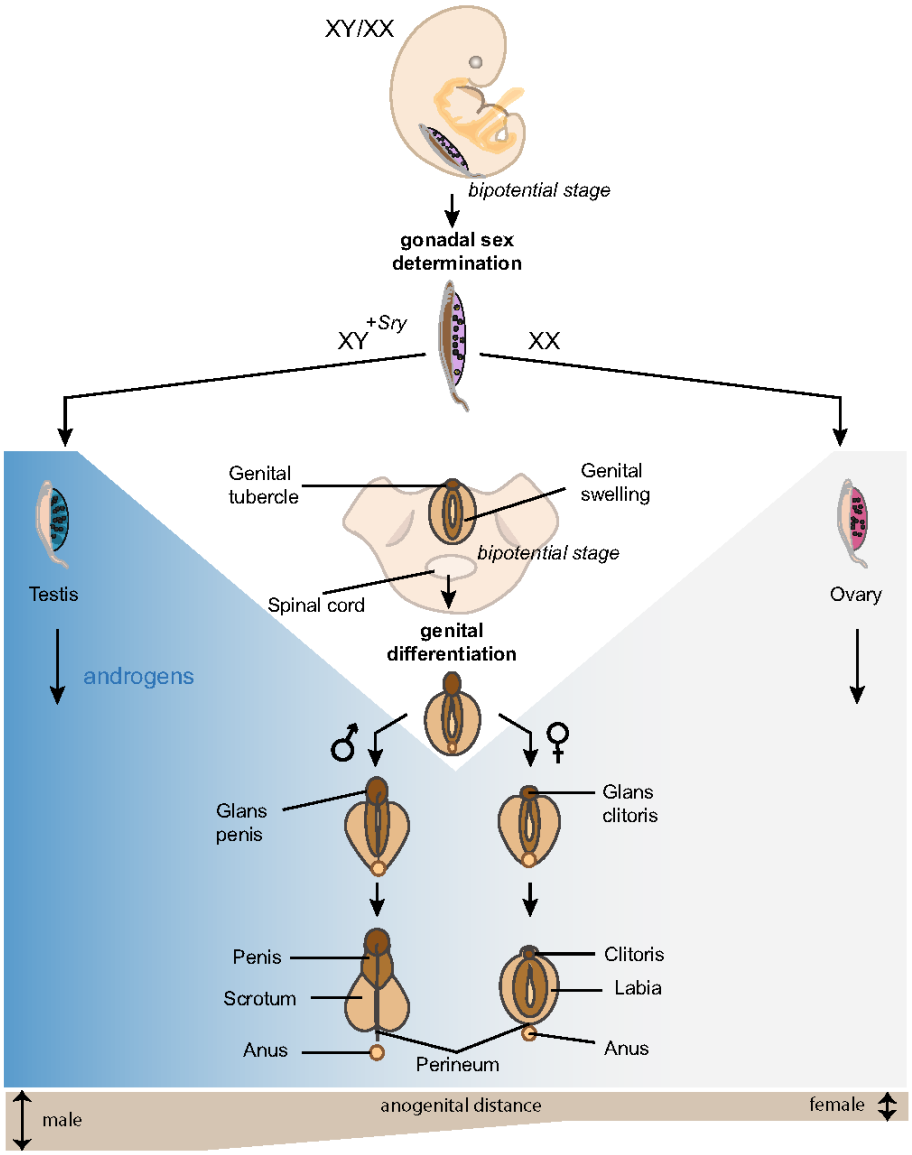

Fig. 1 Development of mammalian external genitalia. Initially, male (XY) and female (XX) fetuses are morphologically indistinguishable. During organogenesis, the bipotential gonadal ridges—located as paired structures running parallel on either side of the body midline—are instructed to differentiate into either testes or ovaries depending on the expression or not of the Y-chromosomal gene Sry in males and females, respectively. This takes place around gestational day 12 in rats and during gestational week 6 in humans. Following gonadal sex determination, the testes will quickly differentiate into compartmentalized structures comprising testis cords and an interstitial space. Steroidogenic Leydig cells appear in the interstitium and start synthesizing androgens—primarily testosterone—that are secreted into the circulation, where they will prompt androgen-sensitive tissue to masculinize. The absence of Leydig cells and androgens in ovaries allows for female differentiation to occur. Locally, in the perineum, testosterone is converted to DHT, which in turn can activate the Androgen receptor and initiate a regulatory cascade that instructs the tissue to develop into male external genitalia, including differentiation of the LABC muscular complex, fusion of the genital swellings to form the scrotum, and growth of the genital tubercle into a penis. From this follows an elongation of the distance between genitalia and anus, referred to as the anogenital distance (AGD). On average, the male AGD is approximately twice that of female and it is a morphometric readout of fetal androgen exposure or activity

often associated with a reduced LABC weight (Christiansen et al. 2008). There are, however, indications that effects on AGD are more complicated than just androgens stimulating muscle growth and anti-androgens that depress this. First, while AGD is thought to be relatively stable throughout life, the perineum is in fact responsive to postnatal changes in androgen levels (Kita et al. 2016; Mitchell et al. 2015). Second, certain EDCs have been found to induce both shorter

Table 1 Summary of rat toxicity studies reporting on AGD measurements following gestational exposure to phthalates

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
Increasing chain length (descending order)					
DMP	750	n.e.	n.e.	n.e.	Gray et al. (2000)
DEP	750	n.e.	n.e.	n.e.	Gray et al. (2000)
DiBP	250	n.e.	5	x	Saillenfait et al. (2017)
	600	14	9	↑	Borch et al. (2006)
	625	22	x	n.e.	Saillenfait et al. (2008)
DBP	500	n.e.	x	x	Scott et al. (2007)
	500	9–14	12(Martino-Andrade)	n.e.Martino-Andrade	Barlow et al. (2004), Howdeshell et al. (2007), Martino-Andrade et al. (2009) and Wolf et al. (1999)
	500	20–28	21(de Mello Sanotos)	n.e.Saillenfait	Carruthers and Foster (2005), de Mello Santos et al. (2017), Mylchreest et al. (1999), Saillenfait et al. (2008), Scott et al. (2008) and Wolf et al. (1999)
	~640	11	10	x	Clewell et al. (2013)
	~650	43	26(AGD/BW)	n.e.	Ema et al. (1998)
	~700	19Increases at other doses	x	n.e.	Lee et al. (2004)
	750	x	9	x	Jiang et al. (2007)
	750	20–24	x	n.e.Mylchreest	Mylchreest et al. (1998) and van den Driesche et al. (2017)
	750	36	x	x	Van den Driesche et al. (2012)
	850	20	x	x	Jiang et al. (2011) and Liu et al. (2016)
	850	x	6	x	Jiang et al. (2015b)
	900	27	x	x	Li et al. (2015)
	1500	48	26(AGD/BW)	n.e.	Ema et al. (2000)
MBuP	750	39	29	n.e.	Ema and Miyawaki (2001)

Table 1 (continued)

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD	References
Increasing chain length (descending order)					
DEHP	30	x	n.e.	n.e.	Christiansen et al. (2009)
	150	n.e.	n.e.	n.e.	Martino-Andrade et al. (2009)
	300	x	5	n.e.	Nardelli et al. (2017)
	500	10	x	x	Howdeshell et al. (2007)
	500	18	18	x	Saillenfait et al. (2009b)
	750	17–18	17(Kita)	x	Jarfelt et al. (2005), Kita et al. (2016) and Lin et al. (2009)
	750	30–34	x	n.e.Gray	Gray et al. (2000) and Wolf et al. (1999)
	900	14	x	x	Christiansen et al. (2010)
	1000	30	11(AGD/BW)	x	Li et al. (2013)
	1500	27	x	n.e.	Moore et al. (2001)
DHP	500	20	23	x	Aydoğan Ahbab and Barlas (2015)
DCHP	~350	6	7	n.e.	Hoshino et al. (2005a)
	500	27	26	x	Aydoğan Ahbab and Barlas (2015)
	750	17	13	n.e.	Saillenfait et al. (2009a)
BBP	500	8–13	x	↑Nagao	Hotchkiss et al. (2004) and Nagao et al. (2000)
	750	9	x	n.e.	Tyl et al. (2004)
	750	30	x	n.e.	Gray et al. (2000)
	1000	38	29	n.e.	Ema and Miyawaki (2002)
MBeP	375	30	29	n.e.	Ema et al. (2003)
DnHP	500	18	18	x	Saillenfait et al. (2009b)
	750	35	31	↓	Saillenfait et al. (2009a)
DiHP	~500	15	x	n.e.	McKee et al. (2006)
DHPP	1000	11	10	n.e.	Saillenfait et al. (2011)
DnOP	1000	n.e.	n.e.	x	Saillenfait et al. (2011)
DOTP	750	n.e.	n.e.	n.e.	Gray et al. (2000)

Table 1 (continued)

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD	References
DiNP	750	n.e.	n.e.	n.e.[Gray]	Clewell et al. (2013) and Gray et al. (2000)
	900	8	6	n.e.	Boberg et al. (2011)
	~1165	n.e.	x	n.e.	Masutomi et al. (2003)
DUDP	500	n.e.	4	n.e.	Saillenfait et al. (2013)
DTDP	1000	n.e.	n.e.	n.e.	Saillenfait et al. (2013)

AGD data after in utero exposure to various phthalates and the dose at which maximum shorter mean AGD was observed. In many instances, percentage shorter AGD was estimated from published graphs, as raw data were not available. A more complete compilation of data is found in Suppl. Table S1

x not assessed; *n.e.* no effect, ↑ longer female AGD or AGDi, ↓ shorter female AGD or AGDi; *DMP* dimethyl phthalate, *DEP* diethyl phthalate, *DBP* dibutyl phthalate, *MBuP* monobutyl phthalate, *DiBP* di-isobutyl phthalate, *DEHP* diethylhexyl phthalate, *DHP* di-*n*-hexyl phthalate, *DCHP* dicyclohexyl phthalate, *BBP* benzyl butyl phthalate, *MBeP* monobenzyl phthalate, *DnHP* di-*n*-hexyl phthalate, *DHPP* di-*n*-heptyl phthalate, *DiHP* di-isoheptyl phthalate, *DnOP* di-*n*-octyl phthalate, *DOTP* dioctyl terephthalate, *DiNP* di-isononyl phthalate, *DUDP* diundecyl phthalate, *DTDP* ditridecyl phthalate

and longer female AGD or have multiple modes of action, as will be discussed later. Thus, more research is needed to fully understand how AGD is affected by fetal endocrine disruption.

Anogenital distance: a biomarker for fetal hormone action and late-life reproductive health

A large number of studies now support various aspects of the 'testicular dysgenesis syndrome' (TDS) hypothesis. However, it has also become increasingly clear that the relationship between hormone disruption and disease outcome is not always obvious. A simple reason for this is that male reproductive disorders can arise from various causes, not least genetic mutations or even genotypic predispositions. This can, in some instances, make it difficult to prove direct cause–effect relationships between chemical exposure and disease state in humans, especially if contributing genetics is present but not characterized. Another major complication is the considerable latency between exposure and disease manifestation, for instance, with regard to reduced sperm quality; a medical condition that can also be influenced by many other factors in the years between fetal life, birth, and adulthood. Hence, a single common biomarker that can predict a number of male reproductive disorders could prove valuable, both from a scientific and clinical point of view.

AGD measurements in human epidemiology and medicine

Fetal exposure to EDCs has been associated with a short AGD in new-born boys. Phthalates are the most frequently reported chemicals associated with a short AGD (Adibi et al. 2015; Bornehag et al. 2015; Bustamante-Montes et al. 2013; Marsee et al. 2006; Suzuki et al. 2012; Swan et al. 2005), but also other compounds including dioxins (Vafeiadi et al. 2013), bisphenol A (Mammadov et al. 2018; Miao et al. 2011) and mild analgesics (Fisher et al. 2016; Lind et al. 2017). Notably, several studies have not found significant correlations between exposure levels and short AGD in boys, including some phthalates (Jensen et al. 2016), dichlorodiphenyl-trichloroethane (DDT) (Bornman et al. 2016), triclosan (Lassen et al. 2016), and various pesticides (Dalsager et al. 2018). These discrepancies do not necessarily diminish the cause for concern, but rather highlight the challenges of obtaining evidence for causal relationships from human epidemiological studies.

With regard to reproductive disorders, many studies have reported significant correlations between short AGD in boys and for instance hypospadias (Cox et al. 2017; Gilboa et al. 2017; Hsieh et al. 2012; Singal et al. 2016),

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Table 2 Summary of rat toxicity studies reporting on AGD measurements following gestational exposure to compounds other than phthalates

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
Drugs					
Acetylsalicylic acid	400	38	x	n.e.	Gupta and Goldman (1986)
Aniline	93	x	20	x	Holm et al. (2015) (mouse study)
Paracetamol	150	9[a]	10.5[a]	x	Kristensen et al. (2011)
	150	x	15	x	Holm et al. (2015) (mouse study)
	350	8	9	x	van den Driesche et al. (2015)
	360	x	n.e.	n.e.	Axelstad et al. (2014)
Dexamethasone	0.1	10	x	x	Van den Driesche et al. (2012)
Finasteride	0.1	x	9	x	Christiansen et al. (2009)
	100	33	x	x	Bowman et al. (2003)
Flutamide	16–20	44[Kita]	41–42	x	Hass et al. (2007); Kita et al. (2016)
	50	16–53	x	x	Foster and Harris (2005) and McIntyre et al. (2001)
	100	33–55	x	x	Mylchreest et al. (1999), Scott et al. (2007) and Welsh et al. 2007)
Ethinyl estradiol	(0.00–0.05)	n.e.	n.e.	(↑)[Mandrup]	Ferguson et al. (2011), Howdeshell et al. (2008) and Mandrup et al. (2013)
Ketoconazole	(50)	n.e.	x	x	Wolf et al. (1999)
	50	8	11	↓	Taxvig et al. (2008)
Pesticides					
Epoxiconazole	3.75	5[a]	5[a]	↑	Hass et al. (2012)
	15	7[(PND0)a]	10[(GD21)a]	↑	Taxvig et al. (2007)
	50	n.e.	n.e.	n.e.	Taxvig et al. (2008)
Myclobutanil	145	12[Increased]	x	x	Goetz et al. (2007)
Prochloraz	(0.01–35)	n.e.	x	(↑)[Melching, Hass]	Christiansen et al. (2009), Hass et al. (2012), Melching-Kollmuss et al. (2017) and Vinggaard et al. (2005)
	150	x	12	↑	Laier et al. (2006)
	250	6	x	↑	Noriega et al. (2005)
Propiconazole	50	n.e.	n.e.	n.e.	Taxvig et al. (2008)
	~ 158	7[Increased]	x	x	Goetz et al. (2007)
Tebuconazole	12.5–50	n.e.	n.e.	(↑)[Hass]	Hass et al. (2012) and Taxvig et al. (2008)
	100	n.e.	10[Increased (only at GD21)]	↑	Taxvig et al. (2007)
Pesticides					
Triadimefon	~ 114	3[Increased]	x	x	Goetz et al. (2007)
Mancozeb	25	n.e.	n.e.	n.e.	Hass et al. (2012)
Vinclozolin	12	n.e.	n.e.	n.e.	Colbert et al. (2005)
	50–60	21[Matsuura]	9–21	n.e.[Matsuura]	Christiansen et al. (2009) and Matsuura et al. (2005a)
	~ 100	28	22	x	Schneider et al. (2011)
	100	28	x	x	Ostby et al. (1999)
	160	x	35	x	Hass et al. (2007)
	200	46–56	x	(↓)[Gray]	Gray et al. (1994) and Wolf et al. (2004)
Procymidone	50	10	9	n.e.	Hass et al. (2012)
	100	24	n.e.	x	Wolf et al. (1999)
	150	x	37	x	Hass et al. (2007)

Table 2 (continued)

Substance	Dose at max effects (mg/kg bw/day)	Male AGD max effect (% shorter)	Male AGDi max effect (% shorter)	Female AGD or AGDi (↑/↓)	References
Linuron	50	8[Not sig. in 2000]	x	n.e.[2002]	McIntyre et al. (2002) and McIntyre et al. (2000)
	75–100	25–31	x	x	Hotchkiss et al. (2004) and Wolf et al. (1999)
p,p'-DDE	100	6–9	x	x	Wolf et al. (1999)
	50–200	x	11 [(AGD/crown–rump length)]	x	Loeffler and Peterson (1999)
Fenitrothion	25	16	x	n.e.	Turner et al. (2002)
Lindane	~16	n.e.	n.e.	(↓)	Matsuura et al. (2005b)
Methoxychlor	~82	n.e.	x	n.e.	Masutomi et al. (2003)
UV filters					
Benzophenone	(~130)	n.e.	n.e.	↓	Hoshino et al. (2005b)
HBM	(~3250)	x	n.e.	n.e.	Nakamura et al. (2015)
OMC	(1000)	n.e.	n.e.	n.e.	Axelstad et al. (2011)
Preservative					
Butylparaben	500	7	6	↓	Boberg et al. (2016)
	600	n.e.	n.e.	n.e.	Boberg et al. (2008)
	1000	16	x	x	Zhang et al. (2014)
Plastic additive					
Bisphenol A	0.25	7	x	↓	Christiansen et al. (2014)
	(0.0025–50)	n.e.	n.e.[(Ferguson, Tinwell)]	n.e.[Ferguson, Tinwell]	Ferguson et al. (2011), Howdeshell et al. (2008) and Tinwell et al. (2002)
	(5–385)	n.e.	x	n.e.	Takagi et al. (2004)
Nonylphenol	(~250)	n.e.	n.e.	↓	Takagi et al. (2004)
Genistein	~67	n.e.	x	n.e.	Masutomi et al. (2003)
Other					
TCDD	0.1	6–12[Not sig. when BW or CR length taken into account]	x	x	Bjerke and Peterson (1994) and Gray et al. (1995)

AGD data after in utero exposure to various substances and the dose at which maximum shorter mean AGD was observed. In many instances, percentage shorter AGD was estimated from published graphs, as raw data were not available. A more complete compilation of data is found in Suppl. Table S1

x not assessed, *n.e.* no effect, ↑ longer female AGD or AGDi, ↓ shorter female AGD or AGDi,*DDE* DDT metabolite, dichloro-diphenyl-dichloro-ethylene, *TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *HBM* 2-hydroxy-4-methoxybenzone, *OMC* octyl methoxycinnamate

[a]Non-monotonic (low-dose) effect

cryptorchidism (Jain and Singal 2013; Jiang et al. 2015a), penile length (Alaee et al. 2014; Thankamony et al. 2014), and sperm quality (Eisenberg et al. 2012; Mendiola et al. 2011). Together, these observations fit the model of a common 'fetal origin of disease', with androgen disruption at the root of the problem. However, they cannot provide definite proof for cause–effect relationships. There is also the complications of accounting for genetic variations of the fetus or maternal characteristics such as parity, factors that themselves can influence the AGD (Barrett et al. 2014; Eisenberg et al. 2013). This ultimately means that two individuals being exposed to the same chemicals could respond differently and display variable degree of changes to the AGD. Because of all these complexities with interpreting human data, the rodent models can be used to provide more robust causative evidence.

AGD measurements in rodent studies

In rats, a short AGD in male offspring after fetal exposure to anti-androgenic compounds often correlates with various reproductive disorders (Bowman et al. 2003; Christiansen et al. 2008; Welsh et al. 2008, 2010). There is evidence to suggest that the more pronounced the effect on the AGD, the more likely additional reproductive defects such as genital malformations are found (Christiansen et al. 2008). However, there is not always a clear correlation between the severity of AGD effects and severity or frequency of other reproductive malformations, such that AGD cannot always stand on its own in the prediction of perceived anti-androgenic effects.

High-exposure studies have reported on male pups with 'female-like' AGD, where male AGD in exposed offspring is

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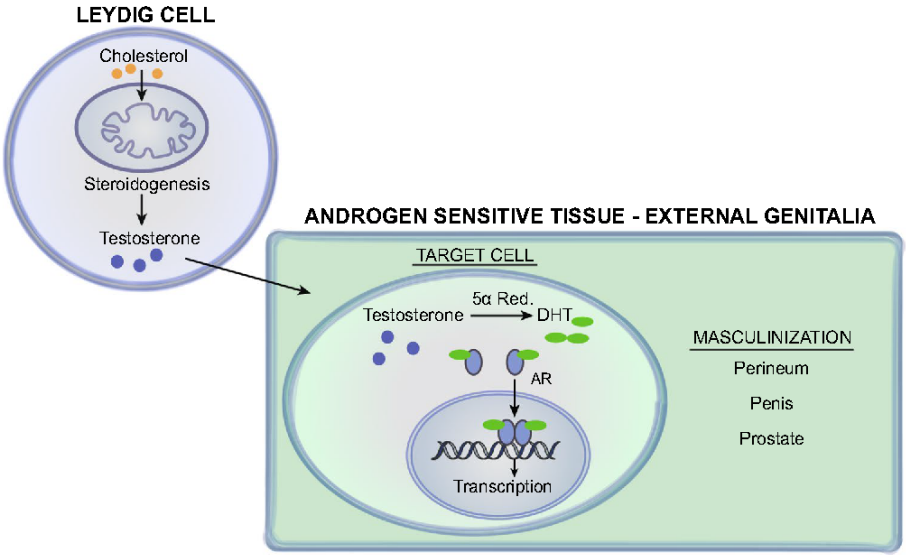

Fig. 2 Androgen signaling cascade required for fetal masculinization. Testosterone is synthesized in the testicular Leydig cells from precursor cholesterol. Testosterone is then secreted into the body circulation, where it acts on different androgen-sensitive tissues and organs. Differentiation of the internal male reproductive accessory organs (e.g., epididymis) is stimulated by testosterone, whereas external genitalia and perineum requires the local conversion of testosterone into DHT by the enzyme 5α-Reductase. DHT can bind to the Androgen receptor (AR) which subsequently locates to the nucleus and regulate expression of target genes. Cell-dependent regulatory signaling pathways are activated and regulate the differentiation into specialized tissues and organs (e.g., LABC complex and external genitalia)

close to 50% that of control males. This does not only make the sex of the offspring difficult to determine (Christiansen et al. 2010; Hass et al. 2007; Ostby et al. 1999; Parks et al. 2000), but also result in additional phenotypes including nipple retention, genital malformations, and reduced reproductive organ weights (Bowman et al. 2003; Christiansen et al. 2008; Welsh et al. 2008, 2010). These reproductive phenotypes are all, at least to some degree, under androgen control during fetal development, making AGD a potentially robust marker for anti-androgenic effects.

AGD in animal toxicity studies: phthalates

The group of compounds most frequently reported to affect male AGD is the phthalate esters. As summarized in Table 1, many different phthalates have been tested in rats, with dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP) being the most prevalent. From an early rat study on DEHP showing testicular toxicity (Gray et al. 1977) and structure–activity relationships suggesting linear side-chain esters of 4–6 carbons to be of specific concern (Foster et al. 1980), numerous toxicity studies on phthalates followed, providing increasing evidence for what later has been termed the 'phthalate syndrome' [(Foster 2006) and Table 1].

Fetal exposure to certain phthalates (chain length C4–C6) results in a short AGD in rat male offspring, without any significant effect on female AGD (Table 1). It is, for the most part, a dose-dependent effect, where increasing dose levels result in progressively shorter AGD. Notably, the magnitude of AGD effects can differ greatly between studies, probably influenced by parameters such as rat strain, group size, or method used for AGD measurements. Body weights may also influence AGD measurements, but are unfortunately not always accounted for. In developmental toxicity studies, the offspring's body weight is in fact frequently affected, particularly at higher exposure levels (Gallavan et al. 1999). Since the AGD correlates with the size of the fetus or newborn pup, body weight should ideally be accounted for by calculating the AGDi, or by including body weight as a covariate in the statistical analysis. Unfortunately, this is not always done, as indicated in Tables 1 and 2. This means that a significantly short AGD in many instances may not be *bona fide* feminization effect, but rather a readout of stunted growth. To remedy this problem for risk-assessment purposes, OECD test guidelines and guidance documents stipulate that bodyweight measurements must be included alongside AGD measurements (guidance documents 151 and 43), as discussed in "AGD measurements in regulatory toxicology".

From the studies listed in Table 1, the most pronounced effect on AGD was seen for DBP at a dose of 1500 mg/kg/day. At this high perinatal exposure level, the average male AGD was 48% shorter than normal, but when accounting for pup body weight, the adjusted value (AGDi) was

26% shorter than normal (Ema et al. 2000). With this in mind, most of the remaining studies investigating the anti-androgenic phthalates have shown maximum shorter male AGD of between 15 and 30% at exposure doses between 100 and 500 mg/kg/day (Table 1). Thus, the active phthalates typically show marked effects on AGD, but rarely complete feminization (i.e., not comparable to female AGD), in a dose-dependent manner. These are high doses that also cause other reproductive disorders and in some cases show signs of systemic toxicity, reduced body weight, or increased liver weights. At lower exposure levels, these same phthalates typically cause less severe, yet statistically significant shorter male AGD, whereas other adverse effects on reproductive organs or systemic toxicity are less prevalent. Hence, a short AGD is often the most prevalent adverse effect observed at the lowest dose, supporting the use of AGD as a sensitive biomarker for these compounds.

Rat Leydig cells appear more sensitive to phthalate disruption than mouse and human Leydig cells (Svechnikov et al. 2016). For instance, the phthalate esters DEHP, MEHP, and DBP reportedly elevate testosterone levels in mice at early developmental stages, but suppress at late gestation, whereas no effects are observed in human fetal testis explants, with or without luteinizing hormone (LH) stimulation. Yet, in cultured mouse Leydig cells, MEHP can both inhibit and stimulate steroidogenesis depending on cell line (Svechnikov et al. 2016). These observations indicate that at least in some instances, disruption to testosterone output is not simply caused by direct disruption of steroidogenesis, or Leydig cells, but likely also includes endocrine signaling networks such as pituitary hormones. The implication of these discrepancies for using AGD as a biomarker for anti-androgenic effects remains uncertain. The best argument would be that since the rat seems to be the most sensitive model, it has the highest potential for safeguarding humans against detrimental effects in the wake of intrauterine exposures. However, more detailed knowledge about molecular events that are both similar and different between species is needed to devise the best test strategies for the future. The human relevance of rodent AGD data could then potentially be confirmed by testing chemicals in human-based cell and tissue systems.

AGD in animal toxicity studies: miscellaneous compounds

As summarized in Table 2, various substances from diverse chemical classes can affect the AGD in rat offspring. These include compounds with a clear anti-androgenic mode of action that cause even more severe effects on AGD than the potent anti-androgenic phthalates.

Prenatal exposure to high doses of certain AR antagonists completely feminizes the male pup AGD to 50% that of control males. This is the case for the pesticide procymidone, vinclozolin, and the non-steroidal prostate cancer drug flutamide (Christiansen et al. 2008; Hass et al. 2007; Ostby et al. 1999; Parks et al. 2000). After exposure to these compounds, the male offspring also displays an increased rate of nipple retention, high incidence of genital malformations, and severely reduced male reproductive organ weights (Bowman et al. 2003; Christiansen et al. 2008; Welsh et al. 2008, 2010). As shown in Table 2, compounds such as the pesticide linuron, or the drugs finasteride and acetylsalicylic acid have also been shown to induce discernibly shorter male AGD (maximum response in the range of 31–38%). Yet, for the majority of the tested chemicals, the maximum effect on AGD in males ranges from 5 to 15% shorter than normal. Of note, for several of the listed compounds the relationship between chemical exposure and AGD does not always follow a continuous pattern, where reduction in the AGD index is proportional to exposure levels, or concomitant reproductive abnormalities.

Fetal exposure to both the antimicrobial preservative butyl paraben (Boberg et al. 2016; Zhang et al. 2014) and the industrial plasticizer bisphenol A (Christiansen et al. 2014) has been shown to shorten the male AGD around 7–16% in the male offspring, albeit there are studies reporting no effects on AGD for both butyl paraben (Boberg et al. 2008) and bisphenol A (Ferguson et al. 2011; Howdeshell et al. 2008; Takagi et al. 2004; Tinwell et al. 2002). In the cases where short AGD was observed with these compounds, clear effects on nipple retention or genital malformations were rarely seen. On the other hand, decreased sperm count or affected prostate development were observed, as were disrupted mammary glands in female offspring (Boberg et al. 2016; Hass et al. 2016; Mandrup et al. 2016). The intuitively appealing explanation for these discrepancies in phenotypic manifestations is that the latter compounds are considered mainly estrogenic. However, they also display weak anti-androgenic potential in vitro (Chen et al. 2007; Reif et al. 2010; Rosenmai et al. 2014; Satoh et al. 2005) and show effect in several in vitro toxicity assays for other mechanisms of action according to Toxicity Forecast [ToxCast; a program developed by the US Environmental Protection Agency to predict hazards and prioritize toxicity testing of environmental chemicals (Dix et al. 2007)]. This leaves the question of whether this small effect on AGD is the result of a weak anti-androgenic or an estrogenic effect, or yet another mechanism of action.

Fetal exposure to the estrogenic compound ethinyl estradiol does not seem to affect AGD in male offspring (Ferguson et al. 2012; Howdeshell et al. 2008; Mandrup et al. 2013), which then argue against the observed effect on male AGD following exposure to butyl paraben or BPA being estrogenic. It appears more likely that there are some weaker anti-androgenic effects exerted by these compounds

that do not increase significantly in efficacy at increasing doses. Another explanation could be lent from observations that estrogenic compounds can reduce the ability of Leydig cells to synthesize testosterone, as reviewed elsewhere (Svechnikov et al. 2010). A recent study where mice were exposed to diethylstilbestrol (DES) also resulted in a short AGD in male offspring, again hypothesized to be caused by reduced testosterone production (Stewart et al. 2018). This is in agreement with what has been observed in rats following exposure to high doses of estrogens, where AR expression is lost in all tissues that show 'anti-androgenic' effects, as well as a reduction in Leydig cell numbers (Williams et al. 2001). Nevertheless, more mechanistic insight is required to fully explain how estrogenic compounds give rise to seemingly anti-androgenic effects.

Another group of chemicals that can elicit effects on reproductive development, including AGD, is the azole fungicides. They are used by both the medical and agricultural industries for their anti-fungal properties. They can, however, provoke side effects in humans and are known to primarily interfere with CYP-family enzymes (Ashley et al. 2006), but also nuclear receptors (Dreisig et al. 2013). As shown in Table 2, there are six azoles that in some studies have been shown to affect the AGD in rats: the drug ketoconazole, and the pesticides epoxiconazole, myclobutanil, prochloraz, propiconazole, and tebuconazole. At tested doses, they do not cause large changes to AGD, but strikingly, many of them cause longer rather than shorter AGD in male offspring. This is not the case for ketoconazole, however, where fetal exposure to 50 mg/kg resulted in around 8–11% shorter male AGD (Taxvig et al. 2008). Notably, a contradictory study has reported no effect on AGD after exposure to ketoconazole at a similarly high dose (Wolf et al. 1999).

Prochloraz is an imidazole fungicide that can cause various adverse effects in rat fetuses at high doses. Regarding reproductive effects, prochloraz exposure can induce nipple retention in male offspring (Christiansen et al. 2009; Vinggaard et al. 2005), whereas effects on AGD are conflicting between studies. If taking birth-weight into account, there were no significant effects on male AGD at doses similar to those causing nipple retention (25–150 mg/kg) (Christiansen et al. 2009; Melching-Kollmuss et al. 2017; Noriega et al. 2005; Vinggaard et al. 2005). Another study reported around 10% shorter male AGD after fetal exposure to high doses of prochloraz concomitant with nipple retention at the same doses (Laier et al. 2006). Screening studies have shown that prochloraz can provoke multiple mechanisms of action in vitro, as it antagonizes the androgen and the estrogen receptor, agonizes the Ah receptor, and inhibits aromatase activity (Vinggaard et al. 2006). Whether or not all these mechanism are activated in vivo, and what effects this would have on the developing fetus, remains to be properly clarified.

Exposure to myclobutanil, propioconazole, and tebuconazole can all seemingly induce longer AGD in male offspring, whereas with epoxiconazole, the picture is less clear with only weak indications that it may affect AGD (Goetz et al. 2007; Hass et al. 2012; Taxvig et al. 2007). By what mechanisms this occur remains unclear. Taken together, however, the azole fungicides seem to elicit different effects on the developing fetus, resulting in effect outcomes not readily explained due to our limited knowledge about mechanisms and modalities, which should be a focus area for future studies.

Note on mild analgesics and their endocrine disrupting properties

Non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol/acetaminophen represent a group of mild analgesics suggested to have endocrine disrupting properties. As recently reviewed (Kristensen et al. 2016), their use across the world has risen dramatically in recent years. Herein, we will not discuss in great detail the potential endocrine effects from the analgesics, but highlight studies where effects on AGD have been reported.

Fetal exposure to therapeutically relevant doses of paracetamol can result in 5–10% reduction in male rat AGDi (Kristensen et al. 2011). Another study using a similar high dose and exposure regimen failed to show a statistically significant change to male AGDi, but here, significant effects on nipple retention and reduction in LABC muscle complex weight were observed (Axelstad et al. 2014). Effects on AGD have also been observed in mice after in utero exposure to mild analgesics, and already in 1986, it was shown that male fetuses exposed to aspirin or indomethacin could present with shorter than normal AGD (Gupta and Goldman 1986). More recently, exposure to both aniline (which is metabolized to paracetamol in vivo) and paracetamol during pregnancy resulted in short AGD in male offspring (Holm et al. 2015). Although these studies strongly suggest that the mild analgesics act as anti-androgenic agents in the male fetuses, there are still not enough mechanistic studies available that convincingly support this conclusion.

Is AGD a useful biomarker for female reproduction?

In females, a longer AGD is considered a masculinization effect, something that would result from the presence of excess androgen levels or ectopic activation of AR. For example, longer than normal female AGD is associated with elevated testosterone levels (Mira-Escolano et al. 2014), exemplified by daughters born of women with polycystic ovarian syndrome (Barrett et al. 2018; Wu et al. 2017).

These clinical observations are further supported by rat studies (Hotchkiss et al. 2007; Ostby and Gray 2004; Ramezani Tehrani et al. 2014; Wolf et al. 2002), and are in line with what we know about how androgens masculinize fetal tissues. However, other mechanisms have also been proposed to underpin masculinization of female fetuses, for instance, ectopic activation of the progesterone receptor (PR).

The fungicide vinclozolin has been suggested to masculinize mouse female fetuses by activation of the PR, further supported by the fact that the synthetic progesterone medroxyprogesterone acetate also masculinizes the female offspring (Buckley et al. 2006). Notably, fetal exposure in rats had no significant effect on female AGD (Hass et al. 2007). Nevertheless, although synthetic progesterones can activate the PR, many synthetic progestins such as medroxyprogesterone also targets other nuclear receptors and can give rise to many off-target effects (Kuhl 2005). For instance, progestins were widely used to prevent miscarriage in the 1950s and 60s, but proved to masculinize the external genitalia of the female offspring (Money and Mathews 1982), and initially referred to as progestin-induced hermaphroditism (Wilkins et al. 1958). Even if these effects seem to be driven by PR-mediated signaling, it cannot be excluded that androgenic modes of action are involved. Not only are several progestins, including medroxyprogesterone, androgenic (Kuhl 2005), but androgen levels can seemingly also be elevated by high levels of progesterone, ultimately giving rise to androgenic effects (Auchus and Chang 2010).

Female rats exposed to the azole fungicide prochloraz present with longer AGD at birth, concomitant with elevated progesterone levels (Laier et al. 2006; Melching-Kollmuss et al. 2017). Prochloraz can induce progesterone synthesis in vitro by CYP17 inhibition at concentrations above 10 nM (Dreisig et al. 2013), a mechanism confirmed in vivo, where fetal plasma concentrations were measured at 24 nM following maternal exposure to 150 mg/kg bw/day of prochloraz (Laier et al. 2006). Although this does not prove that progesterone was acting via the PR to induce masculinization, androgen levels were not elevated in the same animals and hence suggest that this is an alternative mechanism, since azoles themselves do not seem to activate the AR. A definitive answer remains elusive, however, and much work remains to be done on azoles to characterize their sometimes perplexing modes of action.

Estrogens can seemingly also cause longer AGD in females. Although fetal exposure to ethinyl estradiol does not affect male AGD in rats, female offspring can present with longer AGD following exposure to supra-physiologically doses (Casanova et al. 1999; Delclos et al. 2009; Mandrup et al. 2013; NTP 2010; Ryan et al. 2010; Sawaki et al. 2003). Immediately, this effect seems counter-intuitive; however, very high doses of steroidal estrogens can agonize the AR (Vinggaard et al. 1999), which suggests that the effects are driven by androgen action rather than being estrogenic.

There are also examples, where female AGD is shorter than normal following in utero exposure, observations that evoke more questions than answers. The types of compounds capable of shortening female AGD in rodent are also variable and include bisphenol A (Christiansen et al. 2014), butyl paraben (Boberg et al. 2016), ketoconazole (Taxvig et al. 2008), paracetamol (Holm et al. 2016), as well as di-*n*-hexyl phthalate and dicyclohexyl phthalate (Aydoğan Ahbab and Barlas 2015). These studies are not covered in detail herein, and thus not presented with proper weight of evidence as yet any plausible mechanisms- or modes of action are lacking. It would, however, be of great interest to design studies specifically to answer such questions, as they may reveal insights of value to perineal development more broadly.

AGD measurements in regulatory toxicology

AGD in chemical risk assessment

Within a regulatory context, AGD measurements are mandatory to perform at either gestational days 20–21 or postnatal days 0–4 in several OECD test guidelines used to test for developmental and reproductive toxicity in chemical risk assessment. These guidelines include the extended one generation study (TG 443), the two reproductive toxicity screening studies (TG 421/422) and the newly updated TG 414 Developmental toxicity study (OECD 2012, 2016a, b, 2018). The OECD guidance documents (OECD GD 43 and GD 151), which guides the interpretation of these guidelines, states that "A statistically significant change in AGD that cannot be explained by the size of the animal indicates effects of the exposure and should be considered in setting the NOAEL (No Observed Adverse Effect Level)" (OECD 2008, 2013). This means that, when a statistically significant shorter AGD in male rat offspring is considered the critical effect, a NOAEL can be based on this information and used as the point of departure for setting safe exposure levels for humans. Since AGD measurements will be included as an endpoint when performing almost all future regulatory studies investigating developmental and reproductive toxicity, it will improve on the sensitivity for identifying endocrine disruptors and developmental toxicants in mammals. In addition, this inclusion will generate much data, which will contribute to more thorough evaluations of substances and information pertaining to their modes of action.

Other morphological biomarkers to support AGD

Nipples and mammary glands originate from bipotential structures that develop differentially between the sexes in response to specific molecular cues. In humans, both sexes are born with a pair of nipples and breast development in

girls is only initiated during puberty. In rats, the situation is somewhat different, as only the females retain their nipples; postnatal males only possess rudimentary structures (Kratochwil 1971).

In the developing male rodent, the presence of DHT causes regression, or apoptosis of the nipple anlagen (Imperato-McGinley et al. 1986). This process is blocked by fetal exposure to anti-androgens, and these male offspring subsequently display nipples similarly to their female littermates. Therefore, nipple retention in male pups is used alongside AGD as a morphometric marker of impaired androgen action. Although this morphological phenomenon does not occur in humans, it can, nevertheless, be used to predict anti-androgenic effects of chemicals. In other words, the fact that nipples are retained in exposed male rat offspring can be used to predict reproductive disorders caused by chemical exposures, even though nipples do not regress in human males. Whether AGD or nipple retention possess similar sensitivity, as was recently shown for an 18-chemical mixture of anti-androgens (Conley et al. 2018), or if one of the endpoints is more sensitive than the other, seems to depend on what chemical is being tested. Therefore, inclusion of both AGD and nipple retention in reproductive toxicity studies, in a weight-of-evidence manner, can significantly improve on the assessment of potential EDCs (OECD 2015). Nipple retention is also mandatory to assess in three separate OECD test guidelines (TG 443, TG 421/422).

Conclusions and perspectives

AGD has emerged a useful biomarker to detect fetal androgen insufficiency and is now applied in regulatory testing strategies for detecting endocrine disrupting effects. Its utility in a clinical setting is less defined, but it has received more traction in the last few years. It is our conviction that it will remain a standard retrospective biomarker in rodent toxicity studies and risk assessment. In humans, it will increasingly serve as a prospective biomarker, where a shorter than normal AGD in male offspring will be a warning flag for future reproductive complications, not least fertility issues. For this to fully eventuate, however, much more research focusing on answering many knowledge gaps is required. To this end, we would emphasize four areas that we believe should be focused on in the near future.

First, more efforts should be channelled into characterizing the morphoregulatory pathways of perineal development. Although the current dogma stipulates that the length of the AGD is controlled by the level of androgen action during the 'masculinization programming window', other regulatory pathways also seem to play a role, at the very least as effect-outcome modifiers.

Second, there is a need to better define the relationship between anti-androgenic effects and the length of the male AGD, both in rodents and humans. Should we rely purely on 'statistically significant' differences, or should a minimum percentage shorter than control mean be defined? And more importantly, can the magnitude of shortening be used to define magnitude of lost androgen action or future risk of contracting reproductive disease?

Third, to what extent can AGD measurements be used as a stand-alone biomarker to detect anti-androgenic effects in toxicity studies, and where do we need to supplement with additional effect measures? In a weight-of-evidence approach—which is currently recommended—what other measurements should be included, and when? For these evaluations, there should also be stronger emphasis on effect doses and to what extend supra-high doses reliably recapitulates what occurs at more human relevant doses. This latter point seems more important in view of chemicals that do not result in clear monotonic dose–response relationships.

Fourth, can AGD measurements be used as a biomarker in females? Certain perturbations can affect female AGD in either direction, but what does a long or short AGD really measure in female offspring? And is it linked to adverse health effects later in life? Concerning a longer than normal AGD, it would most often, if not always, be a sign of masculinization effects comparable to those observed when female fetuses receives too much androgens. With a shorter AGD, however, the jury is still out.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest pertaining to this work.

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4

Manuscript II

4.1 Manuscript II

Distinct Transcriptional Profiles of the Female, Male, and Finasteride-Induced Feminized Male Anogenital Region in Rat Fetuses.

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Distinct Transcriptional Profiles of the Female, Male, and Finasteride-Induced Feminized Male Anogenital Region in Rat Fetuses

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ABSTRACT

A short anogenital distance (AGD) in males is a marker for incomplete masculinization and a predictor of adverse effects on male reproductive health. For this reason, AGD is used to assess the endocrine disrupting potential of chemicals for risk assessment purposes. The molecular mechanisms underpinning this chemically induced shortening of the AGD, however, remains unclear. Although it is clear that androgen receptor-mediated signaling is essential, evidence also suggest the involvement of other signaling pathways. This study presents the first global transcriptional profile of the anogenital tissue in male rat fetuses with chemically induced short AGD, also including comparison to normal male and female control animals. The antiandrogenic drug finasteride (10 mg/kg bw/day) was used to induce short AGD by exposing time-mated Sprague Dawley rats at gestation days 7–21. The AGD was 37% shorter in exposed male fetuses compared with control males at gestation day 21. Transcriptomics analysis on anogenital tissues revealed a sexually dimorphic transcriptional profile. More than 350 genes were found to be differentially expressed between the 3 groups. The expression pattern of 4 genes of particular interest (*Esr1*, *Padi2*, *Wnt2*, and *Sfrp4*) was also tested by RT-qPCR analyses, indicating that estrogen and Wnt2 signaling play a role in the sexually dimorphic development of the anogenital region. Our transcriptomics profiles provide a stepping-stone for future studies aimed at characterizing the molecular events governing development of the anogenital tissues, as well as describing the detailed Adverse Outcome Pathways for short AGD; an accepted biomarker of endocrine effects for chemical risk assessment.

Key words: anogenital distance; transcriptome; gene array; endocrine disruptors; risk assessment; reproduction.

In mammals, the two sexes are morphologically indistinguishable until the bipotential gonads differentiate into either testes or ovaries in response to genetic cues during early fetal organogenesis (Svingen and Koopman, 2013). Formation of testes leads to the production of androgens that, to a large degree, drive development of the male accessory sex organs as well as general masculinization of the fetus (Jost, 1954). In the absence of high androgen levels, as in females, feminization of the fetus takes place. Thus, the balance between masculinization and feminization of the fetus is largely controlled by androgen signaling.

During development the perineum and surrounding tissues are responsive to androgen signaling. The anlage for the perineal muscles (levator ani/bulbocavernosus [LABC] muscle complex) are present in both sexes, but androgen action prompts the growth and differentiation of the muscles only in the male fetus (Cihak et al., 1970; Ipulan et al., 2014; MacLean et al., 2008). Under normal conditions, the distance between the anus and

the genitals, the anogenital distance (AGD), becomes twice as long in males as in females (Hotchkiss and Vandenbergh, 2005; Salazar-Martinez *et al.*, 2004). The programming of AGD occurs during a short timespan during fetal development (embryonic day 15.5–18.5 in rats and gestation week 8–14 in humans) and is often referred to as the masculinization programming window (MPW) (van den Driesche *et al.*, 2012, 2017; Welsh *et al.*, 2008). If androgen signaling is disrupted during the MPW, it can have severe consequences for androgen-dependent tissues and organs, including the AGD; a morphometric change regarded as a feminization effect (Schwartz *et al.*, 2019).

Because the AGD is so clearly different between the two sexes, and because it is directly dependent upon fetal androgen action, it has emerged as a promising biomarker of incomplete masculinization. Although a short AGD is not necessarily harmful in itself, it is associated with male reproductive disorders in both rodents and humans. This group of disorders is often described as part of the testicular dysgenesis syndrome hypothesis and range from genital malformations at birth (hypospadias and cryptorchidism) to low serum testosterone, low sperm quality, and infertility in adulthood (Skakkebaek *et al.*, 2001). For this reason, measurement of male AGD at birth can be used as a marker to retrospectively evaluate endocrine disruption during fetal life and hence predict future reproductive health outcomes (Schwartz *et al.*, 2019).

The measurement of the AGD is currently incorporated into several OECD test guidelines for reproductive toxicity to test for endocrine disrupting potential of existing and emerging chemicals (OECD, 2016a,b, 2018a,b). It is unclear, however, how various chemicals affect the AGD at the molecular level. Such knowledge could aid in the development for predictive, nonanimal test methods for future risk assessment of chemicals. To start characterizing these mechanisms, we profiled the transcriptome of the anogenital tissues in both male and female rat fetuses, as well as in male fetuses with a short AGD induced by *in utero* exposure to the antiandrogenic prostate cancer drug finasteride, a type II 5α-reductase inhibitor that blocks the conversion of testosterone to dihydrotestosterone (DHT) (Clark *et al.*, 1990; Imperato-McGinley *et al.*, 1992).

MATERIALS AND METHODS

Test compound. The test compound used in this study was finasteride (purity reported by the manufacturer >98%, CAS No.: 98319-26-7). Corn oil was used as control compound and vehicle (product number: C8267-2.5L). Both compounds were purchased from Sigma-Aldrich (Copenhagen, Denmark).

Animal study. Twelve time-mated nulliparous, young adult Sprague Dawley rats with a body weight of approximately 240 ± 30 g were supplied at gestation days (GD) 3 (NTac: SD, SPF, Taconic Europe, Ejby, Denmark). The day of vaginal plug detection was designated GD1. On GD4, dams were distributed into 2 groups of 6 animals with similar body weight distributions. Animals were housed in pairs until GD17 and thereafter individually. The animals were housed under standard conditions in semitransparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU type III, Tecniplast, Buguggiate, Italy) (15× 27×43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden). They were placed in an animal room with controlled environmental conditions: 12 h light-dark cycles with light starting at 9 PM, temperature 22°C ± 1°C, humidity 55% ± 5%, 10 air changes per hour.

All animals were fed a standard diet with Altromin 1314 (soy- and alfalfa-free, Altromin GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast) were provided ad libitum. The PSU bottles and cages as well as the aspenwood shelters (instead of plastic) were used to reduce the risk of migration of bisphenol A that potentially could confound the study results. From GD7 to GD21, dams were weighed daily and dosed by oral gavage by qualified animal technicians with a stainless steel probe 1.2× 80 mm (Scanbur, Karlslunde, Denmark) with either vehicle control (corn oil) or finasteride (10 mg/kg bw/day) at a constant volume of 2 ml/kg bw per day.

Cesarean sections GD21. Dams were decapitated under CO_2/O_2-anesthesia at GD21 and fetuses collected by caesarean section. Uteri were taken out and weighed, and the number of live fetuses, resorptions and implantations were registered. Prior to decapitation of the fetuses, their body weights were recorded. AGD was measured as the distance between the genital papilla and the anus, and measured in all fetuses by the same, blinded technician using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) was calculated by dividing AGD by the cube root of the body weight. The anogenital tissue was isolated from the fetuses by dissection under a stereomicroscope. The tissue was trimmed to include only the genital tubercle, the perineal tissue and the very base of the tail (Supplementary Figure 1). The tissue was placed in RNAlater (Qiagen, Hilden, Germany) and stored at −80°C until further analysis. The animal experiments were carried out at DTU Food (Mørkhøj, Denmark) with ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) and by the inhouse Animal Welfare committee.

RNA extraction and gene array analysis. For the finasteride-exposed males, we selected fetuses with an AGD markedly shorter than control males for RNA analysis. Total RNA was isolated from the perineal tissue ($n = 4$–6 pups/group) using RNeasy mini kits, including on-column DNaseI treatment, according to the manufacturer's instructions (Qiagen, Hilden, Germany). The RNA purity and quantity was analyzed using the Agilent 2100 Bioanalyzer system and Eukaryote Total RNA Nano assay (Agilent Technologies, Santa Clara, California) according to the manufacturer's instructions, and only samples with an RNA integrity number (RIN)-score were included for further analyses except for 1 sample that fell just short of 7. Sample RIN-scores are reported in the sample overview in Supplementary Table 2. The Affymetrix Rat Gene 2.0 ST Array was performed by the Center for Genomic Medicine (Copenhagen University Hospital, Denmark). The gene array data were normalized with the robust multi-array average method (Irizarry *et al.*, 2003) implemented in the statistical software R (version 3.4.2) using the Brainarray custom chip description files so that intensity values are not summarized for each probe set but directly for each Entrez Gene ID (Dai *et al.*, 2005).

Data and software availability. Raw data and transcriptomic signatures were uploaded to the NCBI Gene Expression Omnibus (GEO) (Barrett *et al.*, 2012), to the TOXsIgN (Darde *et al.*, 2018) and to Mendeley-Data repositories under the accession numbers GSE122494, TSP765, and doi: 10.17632/bbtmz7wp4v.1, respectively.

Gene filtration and clustering. Data analysis was performed using the AMEN suite of tools (Chalmel and Primig, 2008). Briefly,

genes showing a signal higher than a given background cutoff (median of the normalized dataset cutoff 6.5483) and at least a 1.5-fold change in at least 1 pairwise comparison were selected as described in Supplementary Figure 2. Gene array quality control assessment data can be found in Supplementary Figure 3. To define a set of 364 genes displaying significant statistical changes across comparisons, the empirical Bayes moderated *t*-statistics was performed using the limma package (*F*-value adjusted using the Benjamini and Hochberg false discovery rate approach, $p < .05$; Smyth, 2004). The resulting genes were then partitioned into 2 broad expression clusters (termed clusters 1 and 2) using the *k*-means algorithm.

Functional analysis. The enrichment analysis module implemented in AMEN was used to identify gene ontology terms (biological processes, molecular functions, and subcellular components) and KEGG pathways significantly associated with each expression cluster by calculating Fisher's exact probability using the Gaussian hypergeometric function (false discovery rate-adjusted *p*-value < .01 number of probes in a given group associated with a given annotation term >5).

Synthesis of cDNA and RT-qPCR analysis. cDNA was synthesized from 500 ng total RNA using a random primer mix (New England Biolabs, Ipswich, Massachusetts) and the Omniscript kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative RT-PCR (RT-qPCR) reactions were run in technical duplicates on an QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, ThermoFischer Scientific) in 11 µl reactions containing: 3 µl diluted (1:20) cDNA, TaqMan Fast Universal Mastermix (2×) (Life Technologies, Carlsbad, California) and TaqMan Gene Expressions Assays (Life Technologies). TaqMan assays were: *Wnt2* (Wingless-type MMTV integration site family member 2, Rn01500736_m1), *Sfrp4* (Secreted frizzled-related protein 4, Rn00585549_m1), *Esr1* (Estrogen receptor 1, Rn01640372_m1), *Padi2* (Protein-arginine deiminase type-2, Rn00568155_m1), *Srd5a2* (5α-reductase, Rn00575595_m1), *Ddx3* (DEAD-box polypeptide 3, Rn04224517_u1), and *Trdn* (Triadin, Rn00572292_m1). Cycling conditions were as follows: An initial step of 95°C for 20 s followed by 45 two-step thermal cycles of 95°C for 1 s and 60°C for 20 s. The relative transcript abundance was calculated using the $2^{-\Delta CT}$ method using *ActB* (β-Actin, Rn00667869_m1) and *Hprt1* (Hypoxanthine guanine phosphoribosyl transferase, Rn01527840) as normalizing genes. Selection of normalizing genes were based on an RT^2 Profiler PCR Arrays (Qiagen, Hilden, Germany) run on the same tissue (unpublished data), where *ActB* and *Hrpt1* showed stable expression.

Statistics. The AGD was analyzed using fetal weight as a covariate and fetal body weights were analyzed using the number of offspring per litter as covariate. For all analyses, the litter was the statistical unit. Statistical analyses were adjusted using litter as an independent, random and nested factor.

Data on weight, dams, AGD and AGDi, were analyzed using one-way ANOVA followed by Dunnett's post hoc test, using the statistical software SAS (SAS Enterprise Guide 6.1, SAS Institute Inc., Cary, North Carolina). For data presentation, group mean ±SEM was calculated from 6 litters/group based on litter means. Statistical analysis of the effect of finasteride on cryptorchidism was done using Fisher's exact test.

Analysis of RT-qPCR data was done by one-way ANOVA with Dunnet's post hoc test using the statistical software GraphPad Prism 5 (GraphPad Software, San Diego, California). In cases of nonequal variance between groups, data were log-transformed prior to one-way ANOVA analysis, whereas the graphs still represent the untransformed data. For data presentation, mean ±SEM was calculated from 4 to 6 pups/group.

RESULTS

Finasteride-Induced Short AGD in Male Offspring

Pregnant Sprague Dawley rats were exposed to 10 mg/kg bw/day finasteride from GD7 to GD21. We assessed maternal body weights, weight gain, and uterine weights of exposed dams for potential maternal toxicity and observed no significant treatment-related differences, nor any signs of maternal toxicity (Supplementary Table 1). At GD21, the body weight of exposed males was lower ($p < .05$) than that of the control males,

Table 1. Litter Data

GD21 Caesarean Section	Control	Exposed
No. of litters	*N*—6	*N*—6
No. of fetuses/litter	14.0±2.4	12.5±2.4
Body weight male (*g*)	4.3±0.4	**3.9±0.3***
Body weight female (*g*)	4.2±0.3	3.6±0.5¤
Male AGD (mm)	3.64±0.2	**2.28±0.1****
Male AGD index	2.24±0.03	**1.45±0.08***
Cryptorchid male fetuses	0	5[a]
Female AGD (mm)	1.81±0.09	1.70±0.11
Female AGD index	1.13±0.04	1.11±0.04

Data represent group means, based on litter means ± SD after exposure GD7-21 (finasteride 10 mg/kg bw/day). Male AGD (mm) was analysed with fetal weight as covariate. AGD index—AGD divided by cube root of body weight. Bold values are statistically different from control.

[a]One litter had 3 cryptorchid males, whereas 2 litters had 1 each, $p = .062$ (Fisher's exact test).

*Significantly different from control, $p < .05$; **Significantly different from Control, $p < .01$. ¤Tends to be different from control, $p = .055$.

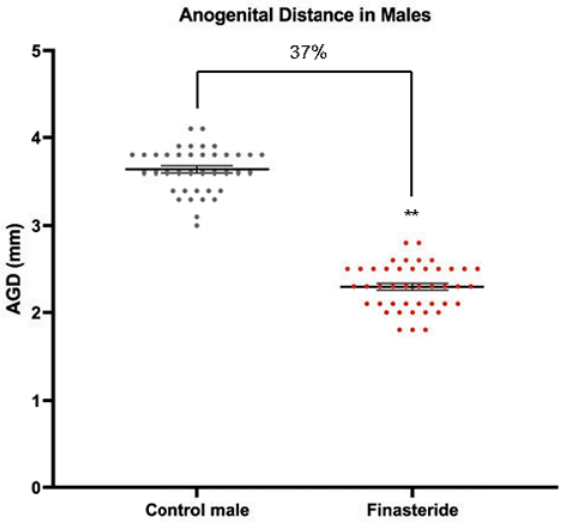

Figure 1. Finasteride-induced short anogenital distance. Anogenital distance (mm) of control males (control), finasteride-exposed males (finasteride, 10 mg/kg bw/day GD7–21). Results are shown as mean±SEM, $n = 6$ litters/group, with each dot representing 1 fetus. **Significantly different from control, $p < .01$ (with bw as covariate).

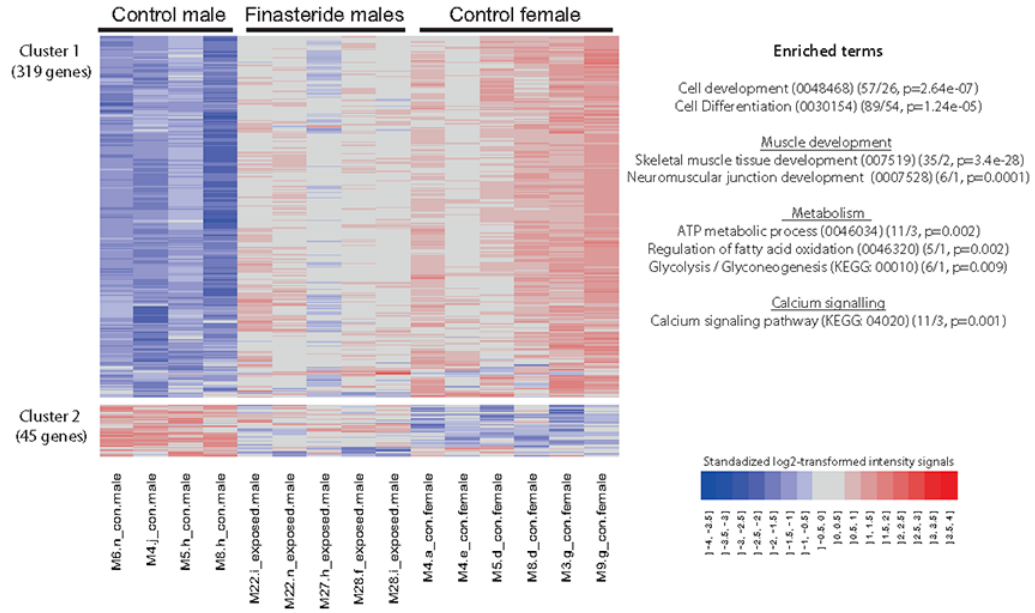

Figure 2. Distinct transcriptome profiles. Heatmap representation of gene expression in the perineal tissues from control males, finasteride-exposed males (finasteride, 10 mg/kg bw/day GD7–21) and control females. Genes (rows) were partitioned according to their expression profiles in the distinct samples (columns) into 2 broad expression cluster containing 319 (cluster 1) and 45 (cluster 2) genes, respectively. The standardized log2-transformed intensity signals are represented in the scale bar. $n = 4$–6 litters/group. The most relevant enriched GO and KEGG terms are indicated at the right side of the heatmap. For each term, the p-value is indicated as well as the number of genes associated with each term and compared with the number of genes that would be expected to be found by chance. See also Supplementary Tables 2 and 4.

whereas the exposed females only tended ($p = .055$) to have a lower body weight than the control females (Table 1). The AGD was on average 37% shorter ($p < .01$) in exposed males when compared with control males (Figure 1). To confirm that this was not a function of lower body weight of the exposed males, AGDi was calculated and was 35% lower ($p < .05$) in exposed males compared with controls. Fetal exposure to finasteride did not affect female AGD or AGDi (Table 1). In addition, 1 litter had 3 cryptorchid male fetuses, whereas 2 litters had 1 cryptorchid male fetus; in total 5 fetuses ($p = .062$).

Distinct Transcriptome Profiles in Control Males, Control Females and Finasteride-Exposed Males

We next performed gene array analysis to investigate the transcriptional profile of the 3 groups. For the group comprising finasteride-exposed males, we selected those with a markedly shorter AGD. A total of 364 differentially expressed genes (DEGs) were identified (Supplementary Table 2) and subsequently partitioned into 2 broad expression clusters (Figure 2). One cluster (cluster 1) comprised 319 genes that were highly expressed in the control females compared with the control males and the second (cluster 2) comprised 45 genes with reversed expression pattern compared with cluster 1. In both clusters, the gene expression profile of the exposed males was intermediary to the male and female control groups. Venn diagrams were made to visualize the up- and downregulated genes between control females or exposed males and control males (Figure 3A), as well as between control males or exposed males and control females (Figure 3B). The exposed males and control females shared approximately 63% of the DEGs (Figure 3A), whereas the overlap in DEGs was only approximately 2% between the exposed males and control males (Figure 3B). Despite exposed males being intermediary to the control males and control females, the exposed males are more closely related to the control females than the control males at the transcriptional level. The lists of genes from the resulting comparisons are available in Supplementary Table 3.

Functional analysis showed no enriched terms for cluster 2. In cluster 1, there were 173 enriched terms (Supplementary Table 4) of which the most relevant biological processes and pathways were related to development, muscle development, metabolism and calcium signaling (Figure 2). These included genes linked to cell development (57 were associated, 26 were expected by chance; 57/26, $p = 2.64e{-}07$), cell differentiation (89/54, $p = 1.24e{-}05$), skeletal muscle tissue development (35/2 $p = 3.4e{-}28$), neuromuscular junction development (6/1, $p = .0001$), ATP metabolic process (11/3, $p = .002$), regulation of fatty acid oxidation (5/1, $p = .002$), the glycolysis/glyconeogenesis pathways (6/1, $p = .009$), and the calcium signaling pathway (11/3, $p = .001$).

Distinct Expression Profiles Validated by RT-qPCR

Among the DEGs, we initially selected 3 genes for further validation by RT-qPCR. These were the Y-chromosome linked DEAD-box helicase 3 (*Ddx3*), 5α-reductase (*Srd5a2*), and Triadin (*Trdn*). The 3 genes were chosen because they were the ones showing the biggest difference in expression levels between the 3 groups (control males, control females, and exposed males). The differences should therefore be detectable in the less sensitive RT-qPCR analysis. *Ddx3* was only detected in males, with expression lower ($p < .05$) in exposed versus control males (Figure 4). *Srd5a2* expression was lower ($p < .01$) in control females compared with control males, whereas *Trdn* expression was higher ($p < .05$) in control females compared with control males

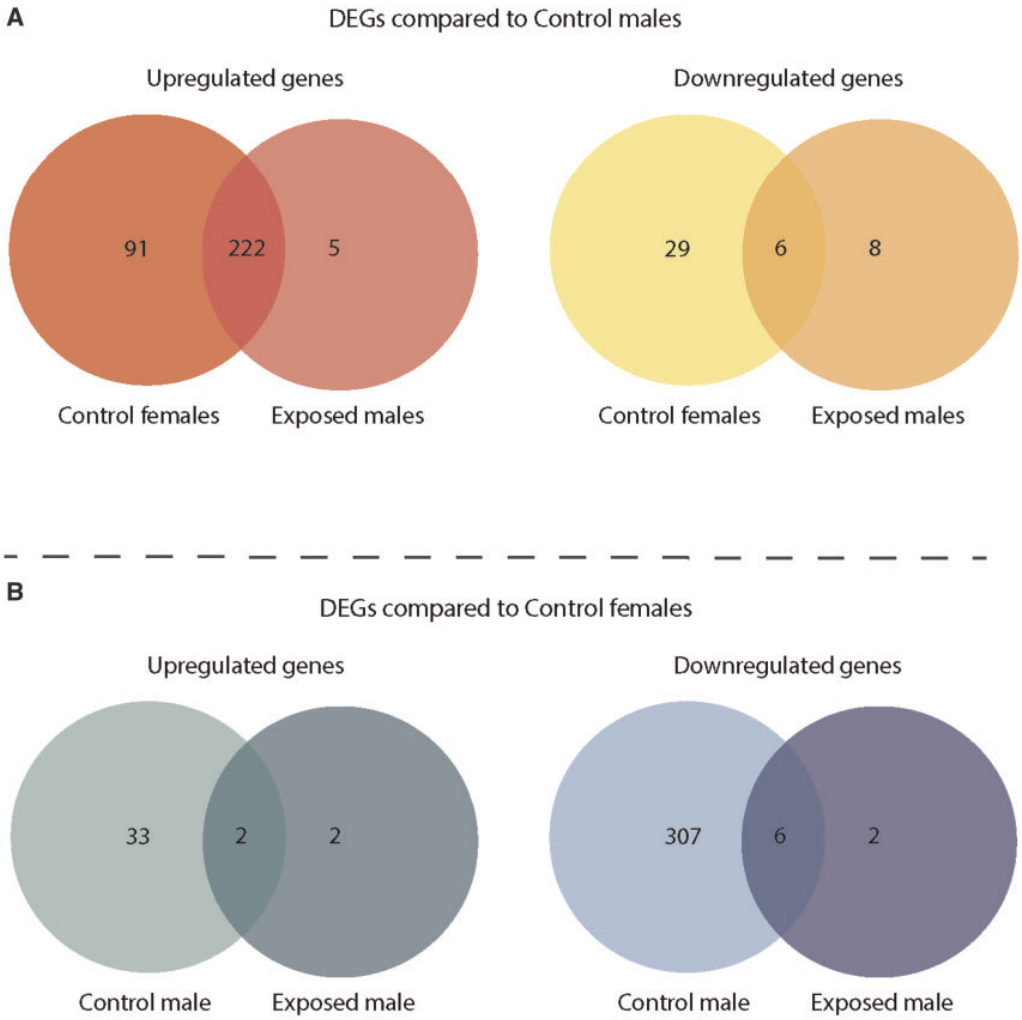

Figure 3. Venn diagrams of differentially expressed genes (DEGs). A, Venn diagrams based on the list of upregulated and downregulated genes when control females and exposed males (finasteride, 10 mg/kg bw/day GD7–21) were compared with control males. B, Venn diagrams based on the list of upregulated and downregulated genes when control males and exposed males (finasteride, 10 mg/kg bw/day GD7–21) were compared with control females. See also Supplementary Table 3.

(Figure 4). These data corroborated the expression pattern observed in the global gene array.

Sexually Dimorphic Expression of Wnt2, Sfrp4, Esr1 and Padi2

We selected another 4 DEGs we believe could be possible key factors in the differential development of the anogenital region based on known functions. In accordance with the gene array data, expression of *Wingless-type MMTV integration site family member 2* (*Wnt2*) (Figure 5), a factor of the Wnt signaling pathway, was significantly lower ($p < .01$) in control females compared with control males. In addition, an inhibitor of Wnt signaling, *Secreted frizzled protein 4* (*Sfrp4*) was higher ($p < .01$) in control females compared with control males.

Expression of *Estrogen receptor 1* (*Esr1*) was higher ($p < .05$) in exposed males compared with control males (Figure 5). This finding was corroborated by the higher expression ($p < .001$) of the *Esr1* regulator *Padi2* in exposed males compared with control males (Figure 5). Expression of *Padi2* was also higher ($p < .01$) in control females compared with control males.

DISCUSSION

In males, a short AGD is an indication of incomplete fetal masculinization. It is associated with adverse male reproductive health effects. Consequently, AGD in fetuses or newborn rodents is used as a biomarker in biological and toxicological research, as well as in regulatory toxicity testing (OECD, 2012, 2016a,b, 2018). Despite its utility in determining antiandrogenicity, we still lack molecular insight into the mechanisms controlling anogenital development and how it relates to the AGD. This hampers the development of potential new, nonanimal test methods for future risk assessment of chemicals.

In this study, we aimed to investigate the transcriptome of the anogenital region in males with short AGD and compare it to that of both control males and control females. We selected a model compound, finasteride, to induce short AGD in male offspring. Finasteride is a synthetic antiandrogen used in humans to treat benign prostatic hyperplasia or prostate cancer and acts by inhibiting type II 5α-reductase, thereby blocking the conversion of testosterone to DHT (Clark *et al.*, 1990; Imperato-McGinley *et al.*, 1992).

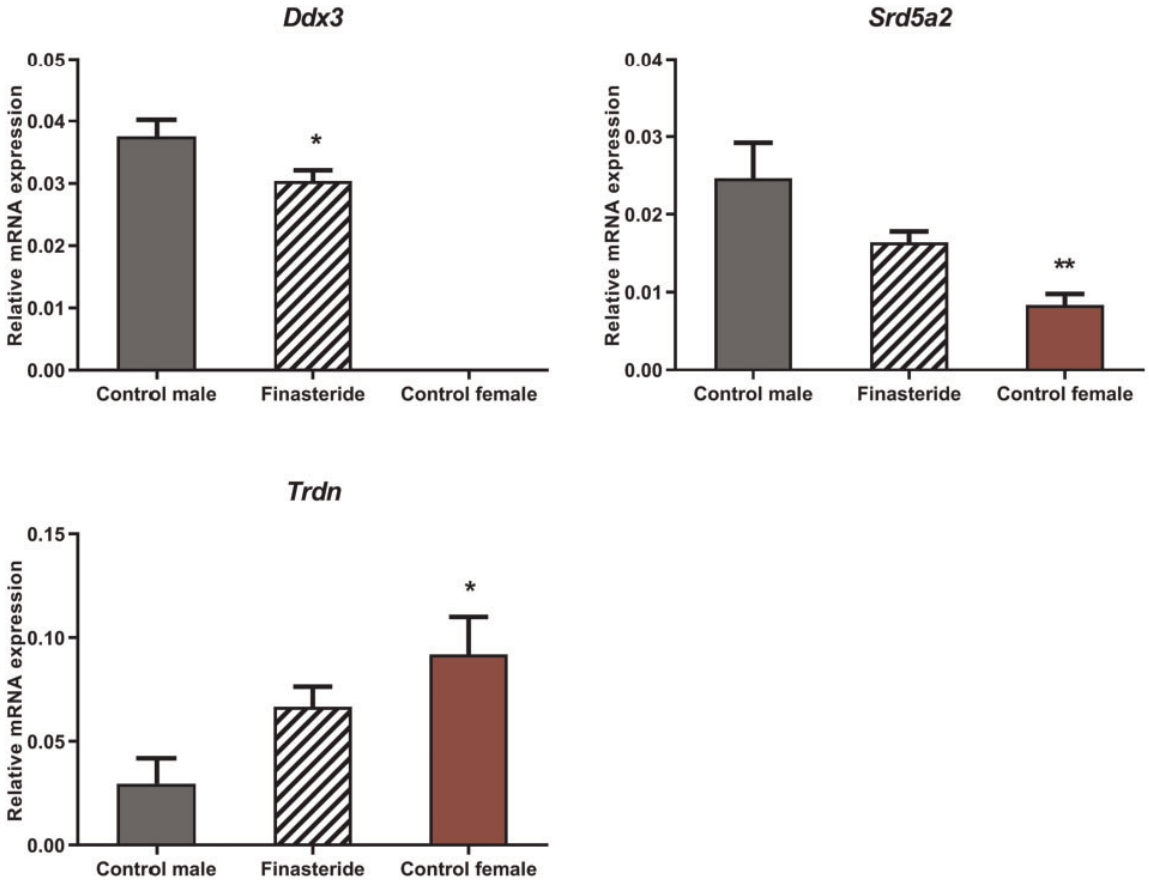

Figure 4. RT-qPCR validation of gene array. RT-qPCR validation of the 3 chosen genes. $n = 4–6$. Results are $2^{-\Delta CT}$ values shown as mean ± SEM. Finasteride: Exposed to finasteride, 10 mg/kg bw/day GD7–21. *Significantly different from control males, $p < .05$. **Significantly different from control males, $p < .01$.

We observed a significantly shorter AGD of 37%, as well as a few male fetuses with cryptorchidism in the exposed group at GD21. These results are consistent with previous *in utero* studies showing several adverse effects on male reproduction after fetal exposure to finasteride (Bowman et al., 2003; Christiansen et al., 2009; Clark et al., 1990, 1993; Hib and Ponzio, 1995; Imperato-McGinley et al., 1992).

With our transcriptomics analysis on tissue from the anogenital region, we found that the control males and control females had distinct transcriptional profiles, whereas the transcriptome of the exposed males with a short AGD were intermediary to that of the 2 control groups. Importantly, comparison of the DEGs showed that the exposed males were more closely related to the control females. The main biological processes affected were related to cell development and differentiation, muscle development, metabolism, and calcium signaling. This corroborates the general view that the development of the anogenital region, and in particular the muscle cells, is sensitive to the masculinizing effects of androgens. It further suggests a general feminization of the finasteride-exposed males, and shows for the first time that short male AGD is associated with marked transcriptional changes in cells of the anogenital region. In addition, RT-qPCR showed that the genes *Ddx3*, *Srd5a2*, *Trdn*, *Padi2*, *Wnt2*, and *Sfrp4* were differentially expressed between control males and control females.

Esr1 expression was higher in finasteride-exposed males compared with control males, but similar to control females. In addition, expression of the *Esr1* cofactor *Padi2* (Zhang et al., 2012) was higher in both exposed males and control females when compared with control males. This suggests that estrogen signaling may play a role in feminization of the anogenital region. In support of this, a polymorphism in the coding region of ESR1 is associated with short AGD in humans (Sathyanarayana et al., 2012). Polymorphisms in ESR1 in humans are associated with hypospadias, cryptorchidism, and reduced fertility (Ban et al., 2008; Safarinejad et al., 2010; Watanabe et al., 2007), disorders that are all thought to be associated with short AGD (Skakkebaek et al., 2001). Furthermore, chemicals with an assumed estrogenic mode of action, such as bisphenol A (Kim et al., 2001; Matthews et al., 2001) and butyl paraben (Byford et al., 2002; Routledge et al., 1998) can induce short AGD in male offspring (Boberg et al., 2016; Christiansen et al., 2014; Zhang et al., 2014), as can treatment with diethylstilbestrol in mouse fetuses (Stewart et al., 2018). Other studies, however, failed to find an effect on AGD following exposure to these two chemicals (Boberg et al., 2008; Ferguson et al., 2011; Howdeshell et al., 2008; Tinwell et al., 2002), a discrepancy that could be due to differences in study design or statistical power. Alternatively, as both chemicals also display antiandrogenic properties *in vitro* (Chen et al., 2007; Rosenmai et al., 2014), the effect on AGD could

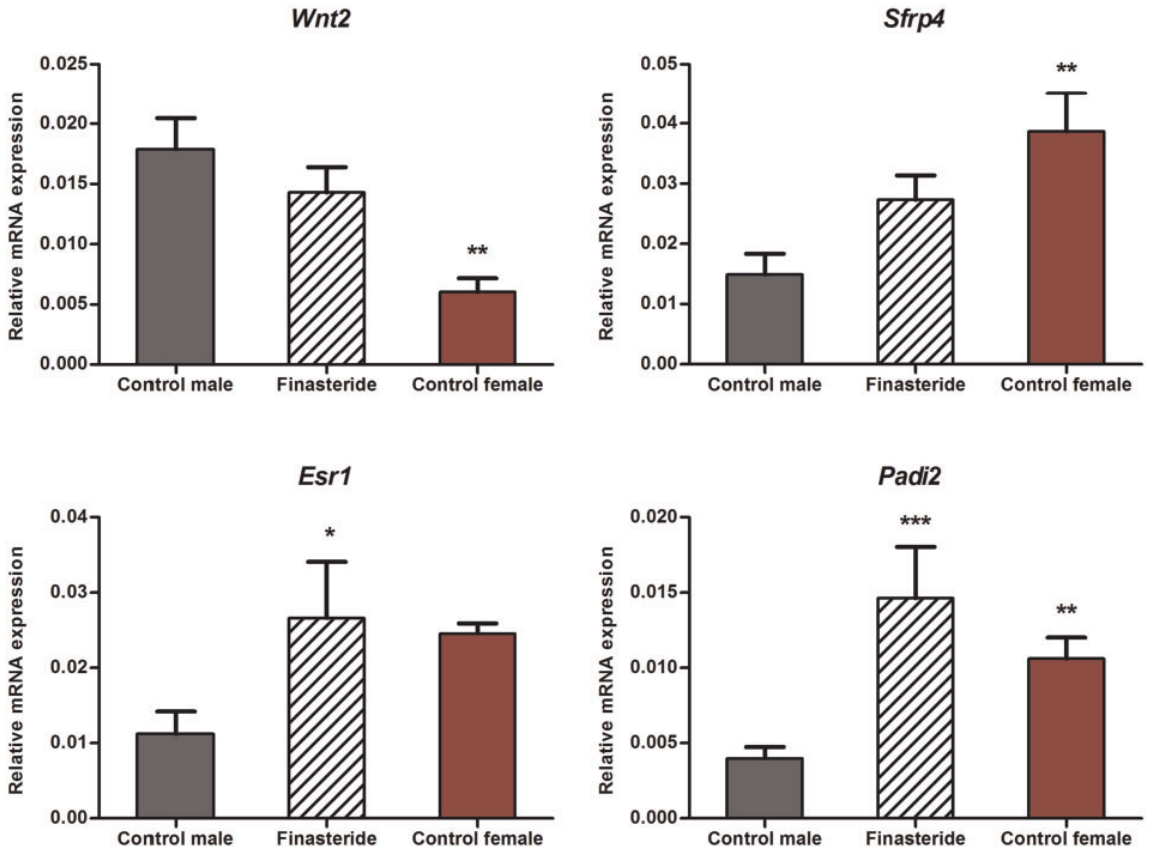

Figure 5. Expression of Wnt2, Sfrp4, Esr1, and Padi2. RT-qPCR validation of *Wnt2*, *Sfrp4*, *Esr1*, and *Padi2*. $n = 4$–6. Results are $2^{-\Delta CT}$ values shown as mean ± SEM. Finasteride: Exposed to finasteride, 10 mg/kg bw/day GD7–21. *Significantly different from control, $p < .05$. **Significantly different from control males, $p < .01$. ***Significantly different from control males, $p < .001$.

instead be antiandrogenic, or disruption to the androgen-estrogen balance, or by other mechanisms still to be determined.

Our gene array revealed that members of the Wnt-signaling pathway are expressed in a sexually dimorphic manner in the rat anogenital region at GD21. Expression of *Wnt2* was higher in control males compared with control females, whereas expression *Sfrp4*, an inhibitor of the Wnt signaling pathway, showed the opposite pattern. To our knowledge, this is the first evidence linking Wnt signaling to anogenital development, albeit expression of *Wnt4* and the Wnt signaling receptor *Fzd4* has been shown to be affected by global knock-out of the *androgen receptor* (*Ar*) in male gastrocnemius muscle (MacLean et al., 2008). Because the perineal LABC muscle complex is highly androgen-responsive (Rand and Breedlove, 1992), it supports the view that finasteride-induced disruption of androgen signaling might also affect Wnt signaling in this muscle, but with Wnt being downstream of AR signaling.

A drawback to our study is that the analysis was performed on GD21 offspring. It is likely that many of the molecular events leading to a sexually dimorphic AGD takes place at an earlier stage and would not be captured in our analysis. As measurements of AGD at early stages such as GD17–19 are impractical, GD21 was chosen to make sure that we had induced short male AGD, as well as allowing for the selection of individual fetuses that had most pronounced shorter AGD relative to controls. This latter point is important in that effects on AGD typically are seen at the population level, but with large individual variation in both exposed and control animals. We therefore selected those GD21 fetuses that presented with obviously shorter AGD and matched them to control fetuses presenting with AGD close to the mean value. We allowed this bias to accommodate our objective of detecting transcripts affected in male fetuses displaying feminized AGD and to circumvent potential issues with larger individual variations at the molecular level were exposed males without a markedly shorter AGD to be included. This inclusion would be necessary if the focus was specifically at elucidating the mechanisms of action of finasteride, whereas this study has a specific focus of detecting differentially regulated transcripts in feminized male AGD more generally. Nevertheless, it is possible to detect sexual differences at the molecular level already at embryonic day 15.5 in mice, corresponding to approximately GD17–18 in rats (Ipulan et al., 2014). Thus, future analyses of the male and female anogenital region at different time points during development will elucidate exactly at which stage during fetal life Wnt and estrogen signaling becomes sexually dimorphic. In addition, future studies on feminized males following chemical exposure should include earlier time points to investigate the early anogenital development, which would require a high number of litters per dose group to account for the variation in AGD. Such analyses may provide further clues as to the role of these pathways in controlling the sex-specific development of the AGD.

In this study, we used the 5α-reductase inhibitor finasteride to induce short AGD in male offspring. This was done specifically to investigate the changes in the anogenital region in response to an androgen signaling inhibitor with a known mechanism of action. Because there is evidence to suggest that other chemicals may affect AGD by other mechanisms (Schwartz *et al.*, 2019) it will be relevant to investigate whether exposure to such chemicals result in similar transcriptional changes in the anogenital region as those we observed in this study, or if the profiles will prove to be different.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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5

Manuscript III

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Expression of estrogen and Wnt related factors in the rat perineal tissues during normal development and following disruption of fetal androgen action

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Supplementary material: Appendix iii

Expression of estrogen and Wnt related factors in the rat perineal tissues during normal development and following disruption of fetal androgen action.

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Abstract

Anogenital distance (AGD) is a biomarker of fetal androgen action. It is used in chemical risk assessment to predict the endocrine disruptive potential of chemicals. Although AGD is directly related to androgen activity during fetal life, it is possible that other pathways play a role in determining AGD, at least as effect outcome modifiers. This could explain the discrepancies sometimes seen between *in vitro* anti-androgenicity and *in vivo* AGD outcomes as well as why chemicals with a non-androgenic mode of action can affect AGD. Based on new and historical data we compared the *in vitro* and *in vivo* anti-androgenic potential of four anti-androgenic compounds (enzalutamide, finasteride, vinclozolin and procymidone) and investigated their effects on perineal gene expression of factors related to Wnt and estrogen signaling pathways. The *in vitro* AR antagonistic potential for enzalutamide was determined using an AR reporter assay (AR-EcoScreen^{TM}). For *in vivo* characterization of the compounds, pregnant Sprague-Dawley rats were exposed via oral gavage from gestational day 7-21. At gestational day 21, concentrations of the four chemicals were measured in amniotic fluid and maternal and fetal plasma, AGD was recorded, and the perineal tissues collected for gene expression analysis of *Wnt2, Sfrp4, Esr1,* and *Padi2*. To assess their temporal expression, the expression of these four genes was assessed in the perineal tissues from male and female rat fetuses at GD15, -17, -18, -20 and -21. Expression of *Sfrp4, Esr1,* and *Padi2* increased over time while *Wnt2* expression was stable. The calculated *in vivo* anti-androgenic potential of the compounds was assessed based on their IC_{50} *in vitro* and the measured concentration in male fetal plasma. We found that, for vinclozolin and procymidone, the calculated *in vivo* anti-androgenic potential of the chemicals did not match the actual observed effects on AGD. While there were indications of cross-talk between androgen signaling and the Wnt and estrogen signaling pathways the discrepancies between the *in vitro* and *in vivo* results was more likely explained by differences in bioavailability of the compounds.

Introduction

In mammals, masculinization of the fetus is largely driven by androgens produced by the testes (Macleod et al, 2010). While gonadal sex determination and initial testis differentiation is androgen independent, all other sex organs and general male phenotypes depend on sufficient levels of testosterone or its catalytic product dihydrotestosterone (DHT) (Macleod et al, 2010; Svingen & Koopman, 2013). Consequently, disruption of androgen signaling, such as suboptimal androgen levels or blockage of androgen signaling through the androgen receptor (AR), will result in incomplete masculinization of the male fetus (Schwartz et al, 2019a; Sharpe, 2020).

A key morphometric measure of fetal androgen signaling is the anogenital distance (AGD). The AGD refers to the distance between anus and external genitalia, thus covering the perineum. High androgen levels in the male fetus prompts the early differentiation and growth of the perineal tissue (Cihak et al, 1970; Ipulan et al, 2014; MacLean et al, 2008). As a result, male AGD is approximately twice as long as female AGD at birth (Hotchkiss & Vandenbergh, 2005; Salazar-Martinez et al, 2004; Schwartz et al, 2019a). Disruption of androgen signaling in the male fetus interferes with perineal development resulting in a short, 'feminized', male AGD (MacLean et al, 2008; Welsh et al, 2008). A short male AGD is thus considered a marker of aberrant fetal masculinization and is associated with reproductive disorders such as hypospadias and cryptorchidism at birth and reduced fertility in adulthood, in both rodents and humans (Dean & Sharpe, 2013; Schwartz et al, 2019a; Thankamony et al, 2009).

AGD is used in several OECD test guidelines for reproductive toxicity to assess if chemicals show endocrine disrupting activities (OECD, 2016a; OECD, 2016b; OECD, 2018a; OECD, 2018b). While many anti-androgenic compounds induce short male AGD in rodent toxicity studies, the measured effect does not always correspond to predicted effects *in vitro* (Gray et al, 2020; Schwartz et al, 2019a). In addition, compounds with a non-androgenic mode of action such as paracetamol also induce short AGD (Kristensen et al, 2011). This suggests that other signaling pathways may contribute to effects on AGD. In addition, it is possible that the effect on AGD also depends on which step in the androgen signaling pathway a given compound targets. We have previously shown that factors of the Wnt and estrogen signaling pathways are expressed in a sexually dimorphic manner in the perineal tissues of rats at GD21 (Schwartz et al, 2019b). In the same study, expression of estrogen related factors was feminized in male fetuses with short AGD induced by exposure to the 5α-reductase inhibitor finasteride. To investigate whether the same feminizing effect on gene expression could be induced by directly blocking the AR rather than diminishing DHT, we

exposed pregnant rats *in vivo* and perineal tissue *ex vivo* to the anti-androgenic prostate cancer drug enzalutamide, a potent and specific AR antagonist (Tran et al, 2009). As additional verification of these pathways being affected by anti-androgenic chemicals, we also included transcriptional analyses of perineum following exposure to two less specific endocrine disruptors, the fungicides vinclozolin and procymidone, that can act as anti-androgens *in vitro* and *in vivo* (Gray et al, 1994; Kelce et al, 1994; Ostby et al, 1999; Scholze et al, In press).

Methods

Test compounds

The test compounds used in the animal study were enzalutamide (purity >98%, CAS No.: 915087-33-1) purchased from Advanced Chemblocks Inc., product no. G-7963, vinclozolin (purity 99.5 %, CAS No.:50471-44-8) purchased from BOC Sciences, USA batch: B16LM02111 and procymidone (purity 99.9%, CAS Nr.: 32809-16-8) from Sigma product no. 36640. Corn oil was purchased from Sigma-Aldrich (Copenhagen, Denmark) and used as control compound and vehicle (product no. C8267-2.5L). For the *ex vivo* study, dihydrotestosterone (DHT) (CAS 521-18-6, product no. D-073, Sigma-Aldrich) was used in addition to enzalutamide and vinclozolin to mimic physiological conditions.

AR-EcoScreen™ assay

The antagonistic effects of enzalutamide on AR were investigated using the Androgen Receptor Stably Transfected Transcriptional Activation Assay (AR STTA) described in OECD test guideline no.458 (OECD, 2016c). Experiments were run with three technical replicates and repeated thrice, with the mean value of technical replicates representing one biological replicate. AR-EcoScreen™ cells (JCRB1328, Japanese Collection of Research Bioresources) were grown in CellBIND® Surface cell culture flasks (Corning® Inc., Corning, New York, USA) in growth medium consisting of Gibco® DMEM/F-12 Nutrient Mixture with L-glutamine and HEPES and without phenol red supplemented with 5 % FBS, 1 % Penicillin-Streptomycin, 200µg/ml Zeocin™ Selection Reagent, and 100µg/ml Hygromycin B (all reagents from Invitrogen™, Life Technologies™, Carlsbad, California, USA). The cells were incubated at 37°C with a humidified atmosphere of 5 % CO_2 and 95 % air. Only cells of passage 3-15 were used for experiments. One day prior to the experiment cells were plated in white 96-well plates (Corning® Inc., Corning, New York, USA) at a density of 9,000 cells/well in assay medium consisting of Gibco® DMEM/F-12 Nutrient Mixture medium, 1 % Penicillin-Streptomycin, with 5% dextran-coated charcoal-treated fetal

bovine serum (DCC-FBS) (all reagents from Invitrogen™, Life Technologies™, Carlsbad, California, USA). The following day the medium was changed to assay medium containing various concentrations of enzalutamide or the control compounds R1881 (known AR agonist, Perkin Elmer) or hydroxyflutamide (known AR antagonist, CAS No. 52806-53-8, Toronto Research Chemicals, Toronto, Ontario, Canada). Enzalutamide was added to the cells in 2-fold serial dilutions ranging from 0.05-12.5 µM. The DMSO vehicle concentrations were constant in all wells. R1881 was added at a concentration of 0.1 nM to all wells when testing for AR antagonism. After cells had been exposed to test compounds for ~20 hours *firefly* luminescence was measured in a luminometer (LUMIstar® Galaxy, BMG LABTECH, Offenburg, Germany) using Dual-Glo®lLuciferase Reagent from the Dual-Glo® Luciferase Assay System from Promega (Madison, Wisconsin, USA). In order to distinguish a decrease in luciferase activity caused by pure antagonism from that caused by cytotoxicity, we measured *Renilla* luminescence using Dual-Glo® Stop & Glo® Reagent from the Dual-Glo® Luciferase Assay System (Promega, Madison, Wisconsin, USA).

Animal studies

Enzalutamide study This study included exposure groups to either finasteride or enzalutamide. The animal study and the data on the finasteride exposed animals have previously been described (Schwartz et al, 2019b) but was also carried out as described below. The control animals in this study and in our previous study are the same. All animal studies were performed at the animal facility of DTU Food in Mørkhøj, Denmark.

Vinclozolin and Procymidone study In each experiment, eighteen time-mated nulliparous, young adult Sprague Dawley rats with a body weight of approximately 255±25 g were supplied at gestational day (GD)3. For the enzalutamide study rats were NTac:SD (SPF, Taconic Europe, Ejby, Denmark) and for the vinclozolin and procymidone study Crl:CD(SD), (Charles River Laboratories, Sulzfeld, Germany). The day of vaginal plug detection was designated GD1. On GD4, dams were randomized and distributed into three groups of six animals with similar body weight distributions. Animals were housed in pairs until GD17 and thereafter individually. The animals were housed under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden, Lynge, Denmark). They were placed in an

animal room with controlled environmental conditions: 12 hr light-dark cycles with light starting at 9 pm, temperature 22 ± 1°C, humidity 55 ± 5%, 10 air changes per hr.
All animals were fed a standard diet with Altromin 1314 (soy- and alfalfa-free, Altromin GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast) were provided ad libitum. The PSU bottles and cages as well as the aspenwood shelters (instead of plastic) were used to eliminate any risk of migration of bisphenol A that could potentially confound the study results. From GD7-21, dams were weighed daily and dosed by oral gavage by qualified animal technicians with a stainless steel probe 1.2x80 mm (Scanbur, Karlslunde, Denmark) at a constant volume of 2 ml/kg bw per day. In the study of Enzalutamide, dams were dosed with either vehicle control (corn oil) or enzalutamide (10 mg/kg bw/day) and in the study of vinclozolin and procymidone dams were dosed with either vehicle control (corn oil), vinclozolin (40 mg/kg bw/day) or procymidone (40 mg/kg bw/day).

Caesarean sections GD 21

Dams were decapitated under CO_2/O_2-anesthesia at GD21 and fetuses were collected by caesarean section. The dams were dosed 1h±15min before decapitation in the same order as caesarean sections were performed. Uteri were taken out and weighed, and the number of live fetuses, resorptions, and implantations were registered. Body weights of the fetuses were recorded prior to decapitation (by a scissor). AGD was measured as the distance between the genital papilla and the anus by the same, blinded technician using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) was calculated by dividing AGD by the cube root of the body weight. Perineal tissues were isolated by dissection under a stereomicroscope and placed in RNAlater for RT-qPCR analysis (Qiagen, Hilden, Germany) and stored at -80 ° C until further analysis.

Temporal expression analyses

Time-mated nulliparous, young adult Sprague Dawley rats were supplied at gestational day (GD)3 and were either NTac:SD (SPF, Taconic Europe, Ejby, Denmark) or Crl:CD(SD), (Charles River Laboratories, Sulzfeld, Germany). Animals were housed under the conditions described above.
The day of vaginal plug detection was designated GD1. Dams were decapitated under CO_2/O_2-anesthesia at GD15, -17, -18 or -20 and fetuses were collected by caesarean section. Perineal tissues were isolated by dissection under a stereomicroscope and placed in RNAlater

for RT-qPCR analysis (Qiagen, Hilden, Germany) and stored at -80 °C until further analysis. GD21 samples were obtained from control animals from the enzalutamide study.
The animal experiments described above were performed under ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) and by the in-house Animal Welfare committee.

Chemical analysis of enzalutamide in vivo

Acetonitrile, formic acid and 25% ammonium hydroxide, all of LC-MS grade, were obtained from Sigma Aldrich, Schneldorf, Germany. Evolute ABN express columns, 30 mg, were purchased from Biotage, Sweden. Water was purified on a Milli Q system, Millipore Corporation, US. First, 50 µl of plasma or amniotic fluid was transferred to an Eppendorf tube and 150 µl icecold acetonitrile was added. The samples were centrifuged at 10,000 x g, 4 °C for 10 min (Ole Dich Instrument makers, Denmark) and the supernatants were passed through an Evolute express ABN column. 20 µl eluate was transferred to an HPLC vial and 80 µl 50% acetonitrile was added prior to analysis by Liquid Chromatography – Mass Spectrometry (LC-MS).
LC was performed on a Dionex Ultimate 3000 RS (Thermo Scientific, CA) with a Poroshell SB C-18 (100x2.1 mm, 2.7 µm particle size) column held at 30 °C (Agilent technologies, Walbron, Germany). The solvent system consisted of A: 2.5 mM ammonium hydroxide + 0.1 % formic acid in water and B: acetonitrile. Solvent programming were: 2% B from 0 to 1 min followed by a linear gradient to 95% B to 14 min, isocratic 95% B from 14 to 16 min followed by reversal to initial conditions to 16.1 min and re-equilibration of the column to 20 min. The flow rate was 0.3 ml/min from 0 to 1 min followed by a linear gradient to 0.4 ml/min to 14 min, which was held to 16 min followed by reversal to initial conditions.
The LC system was connected to a Bruker Daltonics, maXis qTOF mass spectrometer equipped with an electrospray ion source operated in positive ion mode (Bruker Daltonics, Bremen, Germany). The ion source settings were: nebulizer pressure 2 bars, drying gas flow 8 l/min, dry gas temperature 200 °C, capillary voltage 2500 V. The scan range was from 80 to 1000 m/z with an acquisition rate of 2 Hz. Sodium formate dissolved in 50% 2-propanol was introduced in the ion source in a 0.2-0.4 min time segment and used for internal calibration of the data files. Hexakisperflouroetoxyphophazene was used as lock mass calibrant. Matrix matched standard samples were prepared in blank plasma at 6 different levels in the concentration range of: 3 to 10,000 nM. Standards and blanks were analyzed in the beginning of a sequence and after each set of 20 samples. Data files were processed using QuantAnalysis (Bruker Daltonics, Bremen, Germany). Extracted ion chromatograms of m/z

465.1003 ± 0.002 Da were constructed and integrated. Plasma concentrations were calculated based on linear calibrations curves constructed using 1/x weighing.

Ex vivo culture

Fourteen time-mated Sprague Dawley rats were supplied at gestational day (GD)10 (NTac:SD, SPF, Charles River, Germany via. SCANBAR, Karlslunde, Denmark). The day of vaginal plug detection was designated GD1. The animals were housed in ScanTainers (Ventilated Cabinets from Scanbur) with controlled environmental conditions: 12 h light (21.00-9.00 h): 12 h dark (9.00-21.00 h) cycle, humidity 55% ± 5, temperature 22 0 C ± 10 C and ventilation changing air 50-60 times per hour. At GD15 dams were decapitated (guillotined) under CO_2/O_2-anesthesia and fetuses were collected by caesarean section. Tissue sections, including both the perineum and the genital tubercle, were isolated by dissection under a stereomicroscope. They were then cultured for 72h in hanging drops of 50µl media containing either test compound or vehicle (see Supplementary Table S1) and kept at 37 °C, 5% CO_2 and 80% O_2 with media change every 24h. The media used was StemPro™-34 SFM (1X) media (ThermoFischer Scientific, Gibco, cat. no. 10639011) supplemented with 2mM L-glutamine (ThermoFischer Scientific, Gibco, cat. no. 25030-024) and 1% Penicillin/Streptomycin (ThermoFischer Scientific, Gibco, cat. no. 15070063). Enzalutamide and vinclozolin was dissolved in DMSO while DHT was dissolved in absolute ethanol. Both enzalutamide and vinclozolin were tested at the final media concentrations 1µM and 100µM. The DHT controls were exposed to DMSO in the same volume as the enzalutamide exposed group (final concentration of 0.05%). The female controls were exposed to DMSO and ethanol in the same volume as the exposed groups (final concentration of 0.05% each). Chemical solutions were diluted to the final concentration in media, fresh each day. After 72h, the perineal tissues were isolated from the tissue sections by dissection under a stereomicroscope, snap frozen and stored at -80 °C until further analysis. The *ex vivo* animal experiments described above were performed at the DTU Food animal facility (Bio F) in Kgs. Lyngby, under ethical approval from the Danish Animal Experiments Inspectorate (license number 2020-15-0201-00539) and by the in-house Animal Welfare committee.

RNA extraction

Tissues were homogenized in 350µl RLT buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol using 55mm stainless steel balls (Qiagen, Hilden, Germany) and the TissueLyser II system (Qiagen, Hilden, Germany). Total RNA was isolated from the

perineal tissue (n=6 pups/group for the *in vivo* studies, n=3 for the temporal expression analysis and n=10/group for the *ex vivo* study) using RNeasy mini kit (*in vivo* studies) or RNeasy microkit (ex vivo study), including on-column DNaseI treatment, according to manufacturer's instructions (Qiagen, Hilden, Germany).

Synthesis of cDNA and RT-qPCR analysis

cDNA was synthesized from 500 ng total RNA using random primer mix (New England Biolabs, Ipswich, MA, USA) and Omniscript kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantitative RT-PCR (RT-qPCR) reactions were run in technical duplicates on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Thermo Fischer Scientific). The RT-qPCR reactions consisted of diluted cDNA (1:20), TaqMan Gene Expressions Assays (Life Technologies) and TaqMan Fast Universal Mastermix (2X) (Life Technologies, Carlsbad, CA, USA). TaqMan assays were: *Wnt2* (Wingless-type MMTV integration site family member 2, Rn01500736_m1), *Sfrp4* (Secreted frizzled-related protein 4, Rn00585549_m1) *Esr1* (Estrogen receptor 1, Rn01640372_m1), *Padi2* (Protein-arginine deiminase type-2, Rn00568155_m1). Cycling conditions were as follows: an initial step of 95°C for 20 sec followed by 45 two-step thermal cycles of 95°C for 1 sec and 60°C for 20 sec. The relative transcript abundance was calculated using the $2^{-\Delta CT}$ method using *ActB* (β-Actin, Rn00667869_m1) and *Hprt1* (Hypoxanthine guanine phosphoribosyl transferase, Rn01527840) as normalizing genes. Selection of normalizing genes were based on a RT^2 Profiler PCR Arrays (Qiagen, Hilden, Germany) run on the same tissue (unpublished data), where *ActB* and *Hrpt1* showed stable expression.

Statistics

Data from the AR-Eco Screen was analyzed by one-way ANOVA followed by Dunnett's post hoc test in GraphPad Prism 8 (GraphPad Software, San Diego California, USA). Results are presented as mean ± SEM for three independent experiments.

Data on maternal parameters and litter data were assessed for normal distribution by residual statistics. Normally distributed data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

AGD was analyzed using fetal weight as a covariate and fetal body weights were analyzed using the number of offspring per litter as covariate. For all analyses, the litter was the statistical unit. Statistical analyses were adjusted using litter as an independent, random and nested factor.

In vivo data on fetal weight, AGD and AGD index (AGDi, AGD/cube root of bw), were analyzed using one-way ANOVA followed by Dunnett's post hoc test, using the statistical software SAS® (SAS Enterprise Guide 8.1, SAS Institute, Inc., Cary, NC, USA). For data presentation, group mean ± SEM was calculated from six litters/group based on litter means.

One-way ANOVA with Dunnett's post-hoc test was used to analyse RT-qPCR data using the statistical software GraphPad Prism 8 (GraphPad Software, San Diego California, USA). In cases of non-equal variance between groups, data were log-transformed prior to one-way ANOVA analysis, while the graphs still represent the untransformed data. For data presentation, mean ± SEM was calculated from 6-10 pups/group except the temporal expression analysis were n =3 pups/group and here unpaired Student t-test was used for the statistical analysis.

Results

Temporal Expression of *Wnt2, Sfrp4, Esr1* and *Padi2* in the perineal tissues

We first investigated the temporal expression of four genes related to the Wnt and estrogen signaling pathways. Previous studies have shown that these genes are expressed in a sexually dimorphic manner at GD21. For both males and females the expression of *Wnt2* was stable over time (Fig. 1A). In both sexes, the expression of the inhibitor of Wnt signaling, *Secreted frizzled protein 4* (*Sfrp4*) increased after GD15. Expression of *Estrogen receptor 1 (Esr1)* was below the limit of detection in males at GD15. In both sexes, expression of *Esr1* increased over time compared to GD15. Lastly, in females the expression of *Padi2*, a regulator of *Esr1*, was more expressed at GD18 than at GD15.

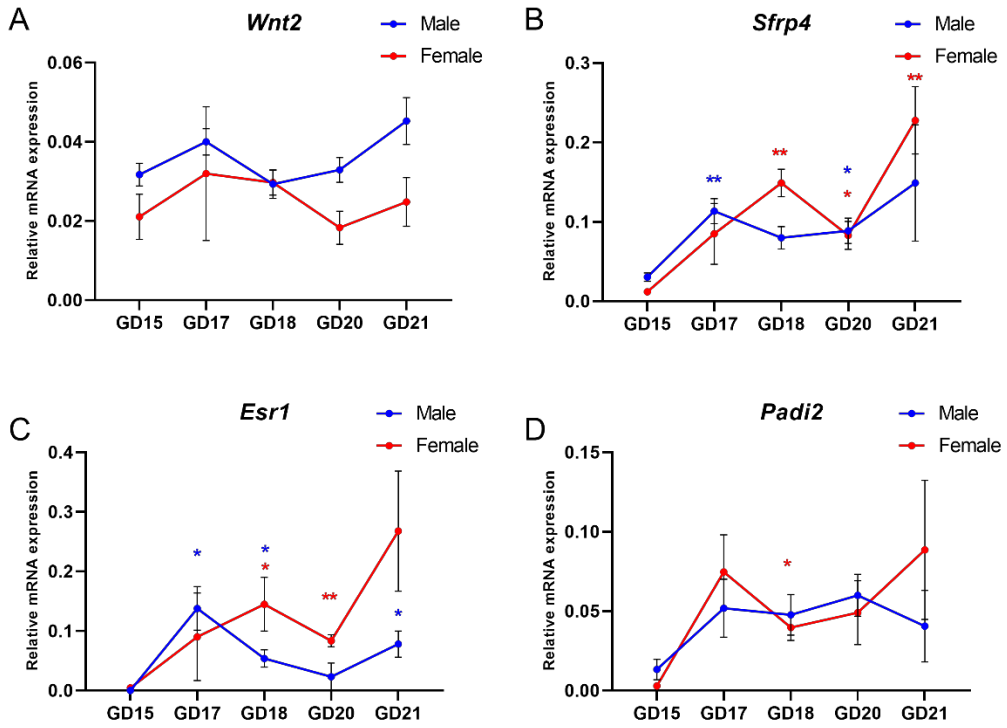

Figure 1: Temporal expression of Wnt and estrogen related factors. Results are $2^{-\Delta CT}$ values shown as mean ± SEM (n=3/group). A: Expression of *Wnt2* is stable over time in both males and females. B, C: Expression of *Sfrp4* and *Esr1* increases over time compared to GD15 in both sexes. D: Expression of *Padi2* is higher in females at GD18 compared to GD15. Results are shown as mean ± SEM, n=3 fetuses/group. *: Significantly different from GD15 within the same sex, $p < 0.05$. **: Significantly different from GD15 within the same sex, $p < 0.01$. Red stars indicate a difference compared to GD15 within females. Blue stars indicate a difference compared to GD15 within males.

Effect of enzalutamide, vinclozolin and procymidone on male AGD

We exposed pregnant rat dams to enzalutamide to investigate the effects of this AR antagonist on AGD and the perineum. This was also done for vinclozolin and procymidone to enable comparisons of different anti-androgens. We first measured maternal body weight, weight gain (GD 7-21), and uterus weight to determine if enzalutamide exposure resulted in maternal toxicity. No significant exposure-related differences between the groups, or any signs of maternal toxicity (Table 1 and Table 2), were observed. In addition, the number of fetuses, as well as fetal weights of both males and females, were similar between the two groups (Table 1 and Table 2). Enzalutamide exposure feminized the male AGD inducing a ~19% shorter AGDi ($p < 0.01$) in exposed males compared to control males (Fig. 2A, Table 1). Vinclozolin and procymidone exposure induced a ~14% and ~17% shorter male AGDi

compared to concurrent male controls ($p < 0.01$), respectively (Fig. 2B, Table 2). There were no significant differences on either AGD or AGDi between exposed females and control females in either of the studies (Table 1 and Table 2).

Table 1 - Pregnancy and litter data for the enzalutamide study

Data represent group means, based on litter means ± SD after oral exposure GD7-21.

ENZ= Enzalutamide, 10 mg/kg bw/day.

	Control	**ENZ**
GD21 Caesarean section		
No. of litters	N=6	N=6
Maternal bw (g), GD 7	262.2 ± 12.2	269.2 ± 11.4
No. of fetuses	14.0 ± 2.4	13.2 ± 2.3
Maternal bw gain GD7- 21 (g)	115.3 ± 18.8	124.5 ± 17.0
Adjusted maternal bw (g)^	289.7 ± 20.0	307.8 ± 18.7
Uterus weight (g)	87.8 ± 10.8	85.9 ± 13.8
Fetal weight male (g) (bw)	4.3 ± 0.4	4.3 ± 0.2
Fetal weight female (g) (bw)	4.2 ± 0.3	4.0 ± 0.3
Male AGD (mm)	3.64 ± 0.2	**2.96 ± 0.1****
Male AGD index	2.24 ± 0.03	**1.82 ± 0.07****
Female AGD (mm)	1.81 ± 0.09	1.67 ± 0.08
Female AGD index	1.13 ± 0.04	1.06 ± 0.07

Values in **bold** are statistically different from control values**,** $p < 0.01$ (**)

^ Adjusted maternal bw (g) is weight of the dams at GD 21 after subtracting the uterus weight

AGD (mm) is analyzed with fetal weight as covariate.

AGD index = AGD divided by cube root of the bodyweight

ENZ: both AGD (mm) in males and AGD index are decreased with 19%

Bw: body weight

Table 2 - Pregnancy and litter data Procymidon and Vinclozolin

Data represent group means, based on litter means ± SD after oral exposure GD 7-21.

PRO= Procymidone, 40 mg/kg bw/day, VIN= Vinclozolin, 40 mg/kg bw/day.

	Control	**PRO**	**VIN**
GD21 Caesarean section			
No. of litters	N=6	N=6	N=6
Maternal bw (g), GD 7	273.4± 22.7	273.1 ± 22.9	272.7 ± 26.1
No. of fetuses	14.2 ± 3.5	14.0 ± 1.5	16.2 ± 2.0
Maternal bw gain GD7- 21 (g)	114.7 ± 23.9	104.5± 8.4	117.0± 6.8
Adjusted maternal bw (g)^	308.9 ± 30.2	284.4 ± 23.9	304.3 ± 19.9
Uterus weight (g)	86.2 ± 18.3	80.9 ± 2.7	93.6 ± 8.9
Fetal weight male (g) (bw)	4.2 ± 0.4	4.1 ± 1.0	4.0 ± 0.2
Fetal weight female (g) (bw)	4.0 ± 0.4	3.9 ± 0.9	3.8 ± 0.2
Male AGD (mm)	3.67 ± 0.14	**3.02 ± 0.30****	**3.10 ± 0.09****
Male AGD index	2.29 ± 0.05	**1.90 ± 0.12****	**1.96 ± 0.09****
Female AGD (mm)	2.01 ± 0.04	1.93 ± 0.07	1.99 ± 0.11
Female AGD index	1.27 ± 0.06	1.24 ± 0.10	1.27 ± 0.08

Values in **bold** are statistically different from control values, $p < 0.01$ (**)

^ Adjusted maternal bw (g) is weight of the dams at GD 21 after subtracting the uterus weight

AGD (mm) is analyzed with fetal weight as covariate,

AGD index = AGD divided by cube root of the bodyweight,

PRO: AGD (mm) in males and AGD index were decreased with 17.7% and 17% respectively

VIN: AGD (mm) in males and AGD index were decreased with 15.5% and 14.4% respectively

BW: body weight

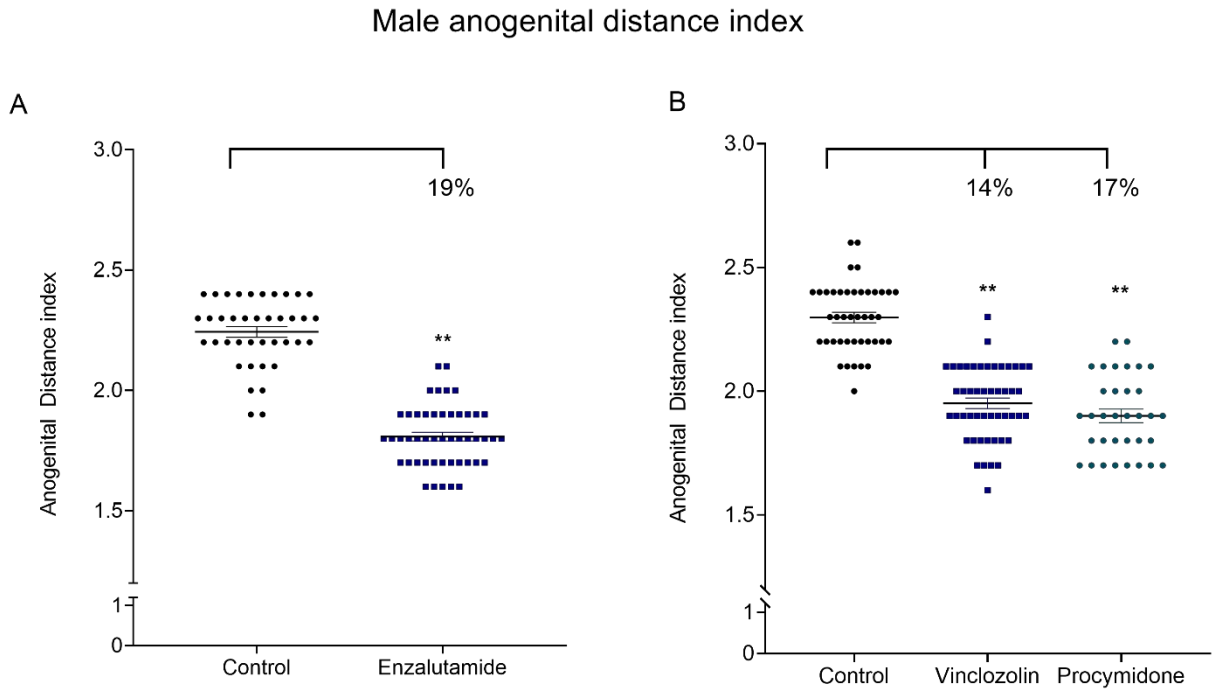

Figure 2: Enzalutamide, vinclozolin and procymidone reduces the male anogenital distance index. Anogenital distance index (AGD/cube root of bw) following exposure to anti-androgenic compounds. **A:** Enzalutamide exposed males had a ~19% shorter AGDi compared to the control males. **B:** Vinclozolin exposed males had a ~14% shorter AGDi compared to control males and procymidone exposed males had a ~17% shorter AGDi compared to control males. Results are shown as mean ± SEM, n=6 litters/group, with each dot representing one male fetus. **: Significantly different from control, $p < 0.01$(with bw as covariate). Bw: body weight.

Effects of disrupted fetal androgen signaling on perineal gene expression

Next, we investigated the transcriptional changes within perineal tissues of exposed males with short AGDi. For this analysis, we used perineal tissues from GD21 offspring from both the enzalutamide study as well as the vinclozolin and procymidone study. In the enzalutamide study, expression of *Wnt2, Sfrp4, Esr1* and *Padi2* were sexually dimorphic as previously shown for these control animals (Fig. 3 A, C, E, G) (Schwartz et al, 2019b). In the vinclozolin and procymidone study, *Wnt2* and *Esr1* were sexually dimorphic. Here, the control males had a higher expression of *Wnt2* ($p < 0.05$) and a lower expression of *Esr1* compared to control females (Fig. 3 B, F). Expression of *Sfrp4* and *Padi2* was not statistically significant between control males and control females in this study (Fig. 3 D, H). Exposure to enzalutamide *in utero* did not affect expression of *Wnt2* but caused a statistically significant increase in *Sfrp4* exposed males compared to control males (Fig. 3 A, C). Although *Esr1* expression in the exposed males was intermediary to that of the control

male and control female, it was not statically significant different (Fig. 3 E). For *Padi2*, a large variation within the exposed groups was observed and there were no statistically significant difference between *Padi2* expression in exposed males and control males (Fig. 3 G). In males exposed *in utero* to either vinclozolin or procymidone, the mean expression of *Wnt2, Sfrp4, Esr1* and *Padi2* was consistently intermediary to that of the control males and control females. We did, however, not find any statistically significant changes in expression in any of the 4 investigated genes (Fig. 3 B, D, F, H).

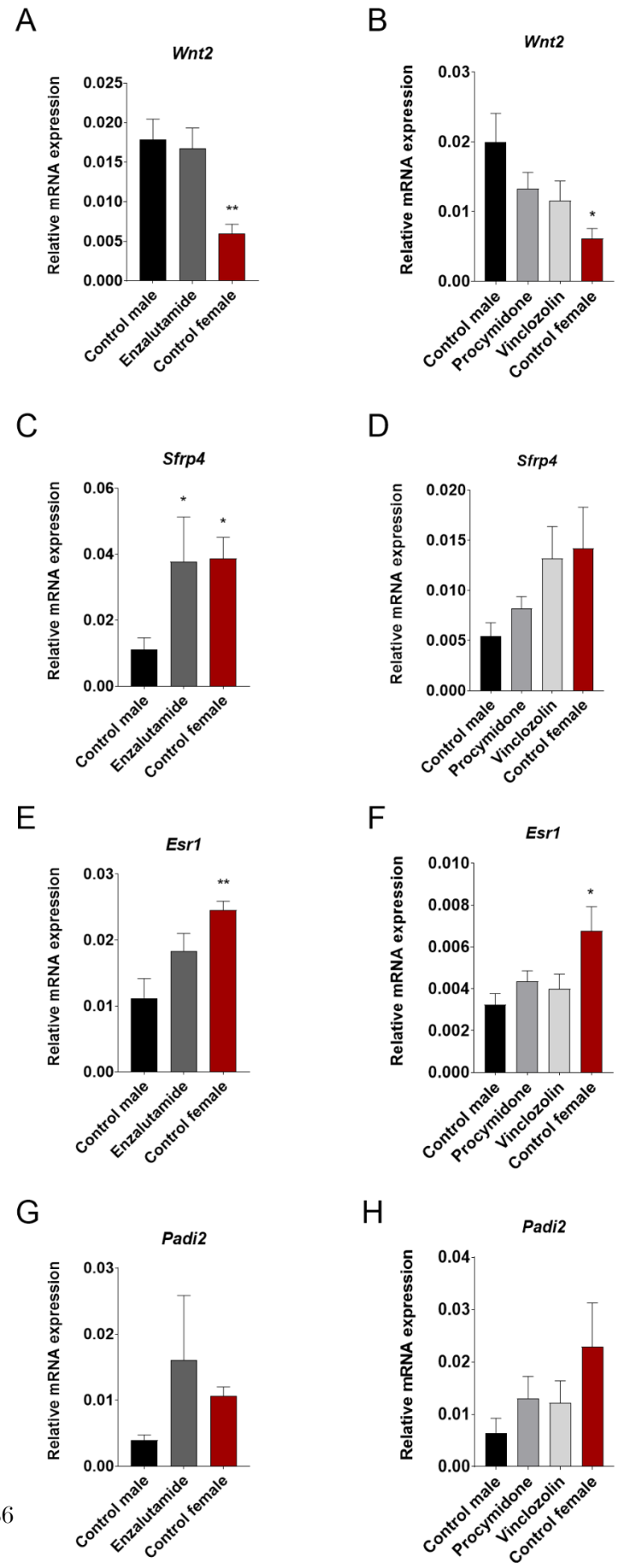

Figure 3: Perineal gene expression following enzalutamide, vinclozolin and procymidone exposure.
RT-qPCR of Wnt and estrogen related genes. Generally, a difference in expression levels of the four genes was seen between male and female control animals. A pattern of lower expression of *Wnt2*, higher expression of *Sfrp4* and *Esr1* and seemingly a higher expression of *Padi2*, however not significant, was seen in both studies.
A, C, E, G: Exposure to enzalutamide caused a significant upregulation of *Sfrp4* in exposed males.
B, D, F, H: Exposure to vinclozolin and procymidone did not significantly affect gene expression.
Results are $2^{-\Delta CT}$ values shown as mean ± SEM (n = 6 / group, *: Significantly different from control males, $p < 0.05$. **: Significantly different from control males, $p<0.01$.)

Ex vivo culture of perineum

We set up *ex vivo* cultures of the perineum in order to get controlled culture conditions and direct exposure of the tissue to the anti-androgenic chemicals. The expression of *Wnt2*, *Sfrp4*, *Esr1* and *Padi2* was similar in male tissue exposed to DHT (control conditions), male tissue exposed to DHT + enzalutamide or to DHT + vinclozolin and female tissue (Fig. 4).

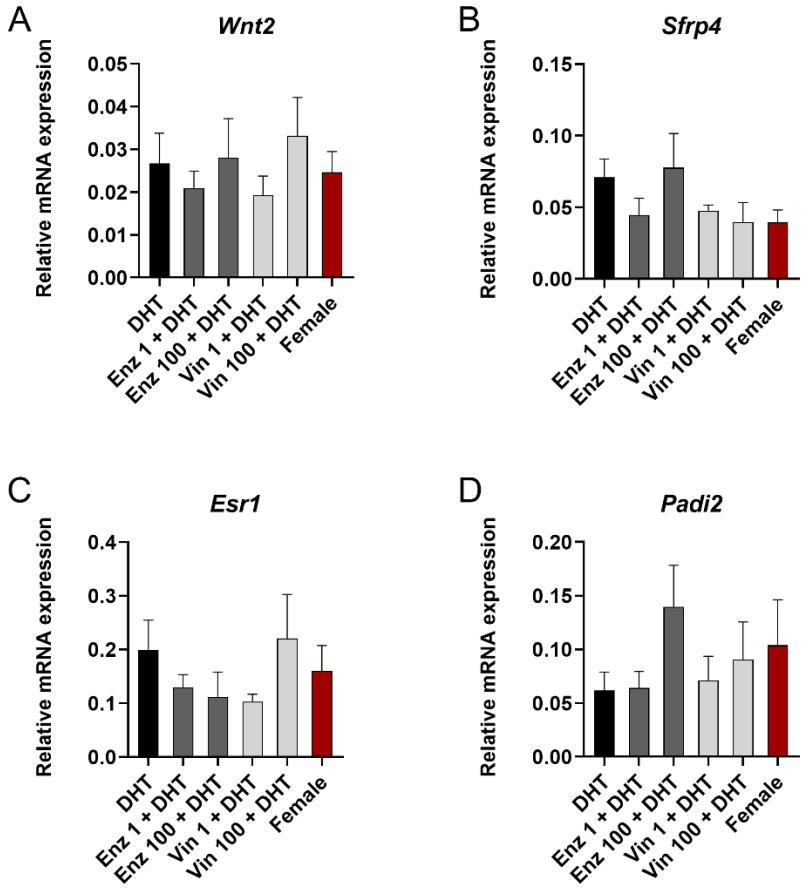

Figure 4: Perineal gene expression following enzalutamide exposure in *ex vivo* culture. RT-qPCR of Wnt and estrogen related genes. Perineal tissue was cultured *ex vivo* for 72h being exposed to 1 or 100 µM of either enzalutamide (Enz) or vinclozolin (Vin). All male cultures were exposed to 1 nm of dihydrotestosterone (DHT). A-D: Expression of *Wnt2, Sfrp4, Esr2* and *Padi2* was not different between male and female tissue and was not affected by the chemical exposure. Results are $2^{-\Delta CT}$ values shown as mean ± SEM (n=6-10/group).

In vitro potency of enzalutamide

To be able to directly compare data from the enzalutamide study with those of finasteride, vinclozolin and procymidone, we assessed enzalutamide using the AR-EcoScreen™ assay (OECD, 2016c). Enzalutamide showed AR antagonistic activity at all concentrations

($p<0.001$) between 0.05-12.5 µM (Fig. 5A). The lowest observed effect concentration (LOEC) was 0.05 µM while the IC_{50} value was 0.1 µM. We confirmed that the reduction in luciferase activity was not due to cytotoxicity following enzalutamide exposure (Fig. 5B). Furthermore, enzalutamide did not show any agonistic activity (Fig. 5C).

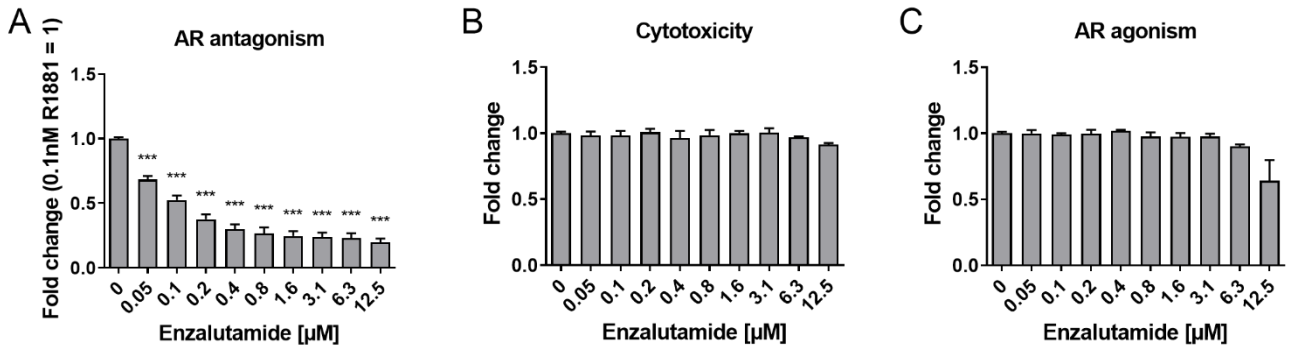

Figure 5: Enzalutamide antagonized the AR *in vitro*. Dose-response curves for enzalutamide in the AR-EcoScreen™. **A**: Cells were incubated with increasing concentrations of enzalutamide (µM) in the presence of 0.1nM R1881. The values are presented as fold change in firefly luminescence relative to the response achieved with 0.1nM R1881, which is set to 1.. **B**: No cytotoxicity was seen following enzalutamide exposure. The values are presented as fold change in *Renilla* luminescence. **C**: Cells were incubated with increasing concentrations of enzalutamide (µM). The values are presented as fold change in firefly luminescence. The values are presented as mean ± SEM (n=3), $p<0.001$ = ***)

In vivo concentrations of enzalutamide

We determined the internal exposure of enzalutamide in various biological compartments *in vivo* (i.e. maternal plasma, amniotic fluid and male and female fetal plasma) at GD21. In maternal plasma, the concentration was 1002 ± 102nM, while it was 285 ± 33nM in amniotic fluid, and 282 ± 41nM and 122 ± 15nM in the plasma of the female and male fetuses, respectively (Fig. 6A).

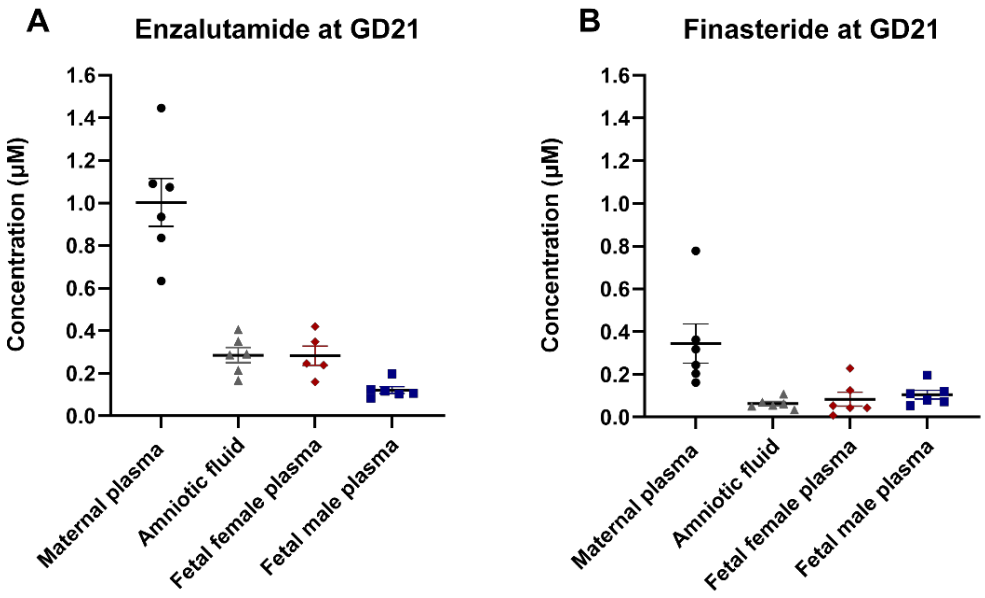

Figure 6: Enzalutamide and finasteride levels in plasma and amniotic fluid. Concentration (µM) of enzalutamide (**A**) and finasteride (**B**) in maternal plasma, amniotic fluid or plasma of the male and female fetuses at GD21 following exposure of pregnant rats from GD7 to GD21 to 10 mg/kg bw/day of enzalutamide or finasteride. Results shown are mean ± SEM of pooled samples from 6 litters.

Effective in vivo inhibition

We gathered present and previous data on enzalutamide, finasteride, vinclozolin and procymidone to enable comparisons of the potencies of these chemicals (Table 3). In combination with the *in vitro* IC_{50} values for each compound, we estimated the *in vivo* anti-androgenic activity for each compound. Finasteride has an IC_{50} for the 5α-reductase of 0.004 µM (FDA, 2011). The IC_{50} of enzalutamide for AR is 0.1 µM while for procymidone it is 0.4 µM (Scholze et al, In press). Vinclozolin is metabolized *in vivo* to the metabolites M1 and M2 with an IC_{50} of 2.0 µM and 0.2 µM, respectively (Vinggaard et al, 2008). The average concentrations of enzalutamide and finasteride in the male fetuses at GD21 were 0.1 µM and 0.8 µM, respectively (Figure 6A, B). While the parent compound could not be measured at GD21, the concentration of M1 were 18 µM and M2 3.4 µM, and for procymidone it was 7.8 µM (Scholze et al, In press). Based on IC_{50} and the concentration of in the male fetal plasma, finasteride is expected to inhibit ~100% of 5α-reductase activity. Likewise, procymidone and vinclozolin are expected to block ~100% of AR activity, while the expected block of enzalutamide is only ~50%.

Table 3 – Compound comparison

		In vitro	*In vivo*				
Compound	**Mechanism**	**IC_{50} (µM)**	**Dose (mg/kg bw/day)**	**Concentration in male fetal plasma at GD21 (µM)**	**Amount transferred to the male fetus at GD21 (%)**	***In vivo* inhibition based on IC_{50} and the fetal plasma concentration (%)**	**AGDi at GD21 (% reduction)**
Enzalutamide	AR antagonism	0.1	10	0.1	1	~50	19
Finasteride	5α-reductase inhibition	0.004[a]	10[b]	0.8	8	100	35[b]
Vinclozolin	AR antagonism	0.3[c]	40	Below limit of quantification[c]			14
- M1		2.0[d]		M1: 18[c]	45	100	
- M2		0.2[d]		M2:3.4[c]	8.5	100	
Procymidone	AR antagonism and effects on steroidogenesis	0.4[c]	40	7.8[c]	19.5	100	17

[a]: IC_{50} for the human Type II 5α-reductase isozyme. Data from (FDA, 2011)

[b]: Data from (Schwartz et al, 2019b)

[c]: (Scholze et al, In press)

[d]: Data from (Vinggaard et al, 2008)

Discussion

Despite the frequent use of AGD measurements in toxicological studies, the molecular mechanisms by which chemicals interfere with perineal development to induce short AGD remains poorly characterized (Schwartz et al, 2019a). This is in itself not an issue when performing *in vivo* toxicity studies, since the adverse outcome would be measurable independent of modes of action. It is a potential challenge in a new toxicity testing paradigm, however, where non-animal test assays are meant to replace animal studies. In this scenario, we need enough knowledge about mechanism of action to be able to predict apical endpoints based on alternative test method data only.

We have previously shown a sexually dimorphic expression of factors of the Wnt and estrogen signaling pathways in the perineum of rat fetuses at GD21 (Schwartz et al, 2019b). It is, however, likely that the molecular changes within the perineum, resulting in a short

AGD, takes place earlier than GD21. Masculinization of the fetus takes place in a limited time window referred to as the masculinization programming window (MPW), ranging from embryonic day (e)15.5 to e18.5 in rats corresponding to gestation week 8-14 in humans (van den Driesche et al, 2017; van den Driesche et al, 2011). When androgen signaling is disrupted within this time window a shorter AGD is seen in male offspring (Welsh et al, 2008). In addition, sex specific differences in the myogenic marker MyoD can be detected in the perineum already at e15.5 in mice corresponding to GD17 in rats (Ipulan et al, 2014). In agreement with this and the timing of the MPW, our preliminary analysis of the temporal expression of *Sfrp4, Esr1* and *Padi2* show that they are similar between males and females until at least GD17. However, as this study was based on only three fetuses in each group and very high variation was seen, much more work is needed before conclusions can be drawn.

At GD21, we found the average AGDi to be ~19% shorter in males exposed *in utero* to enzalutamide than in control males. To our knowledge, enzalutamide has not previously been used as a model compound in reproductive toxicity studies. One previous report, but no peer-reviewed papers, have tested the ability of enzalutamide to affect male AGD; and although this study report shorter male AGD in exposed offspring, it does not report on how much shorter compared to controls (TGA, 2014). A short AGD is nevertheless in line with what would be expected based on the IC_{50} for AR antagonism and the obtained plasma concentration in the male fetus. In comparison, finasteride which, based in the IC_{50} and the fetal plasma concentration, is expected to completely inhibit the *in vivo* activity of the 5α-reductase, induces 35% shorter male AGDi (Schwartz et al, 2019b). This is close to maximal observed effects on AGDi of about 40% induced by the AR antagonist flutamide and high doses of vinclozolin and procymidone (Gray et al, 1994; Hass et al, 2007; Kita et al, 2016; Wolf et al, 2004). Based on the IC_{50} values obtained *in vitro* for each compound and the internal concentrations in male fetuses, it is clear that finasteride has a higher anti-androgenic potential *in vivo* compared to enzalutamide which explains the differences in AGDi outcome.

In contrast to enzalutamide, several studies report a short male AGD or AGDi following *in utero* exposure to vinclozolin and procymidone (Christiansen et al, 2009; Gray et al, 1994; Hass et al, 2012; Hass et al, 2007; Matsuura et al, 2005; Wolf et al, 1999). Notably, based on the $IC_{50,}$ AR is expected to be completely inhibited at the fetal plasma concentrations of vinclozolin and procymidone, but the AGDi is only reduced by ~15%. We know from previous studies that higher concentrations of procymidone and vinclozolin can induce effects on AGD or AGDi of up to 30-50% reduction (Gray et al, 1994; Hass et al, 2007; Wolf et al,

2004). This indicates that the inhibition of AR determined in the *in vitro* AR reporter assay is not always sufficient to predict the AGD outcome. Indeed, a recent analysis of data on more than 50 different chemicals from 250 studies indicates that the concordance between *in vitro* anti-androgenicity and AGD outcome was only ~50% (Gray et al, 2020). In the present study, we have measured the fetal plasma concentrations as a way to account for parameters such as absorption and excretion. Furthermore, we have included data for the two vinclozolin metabolites M1 and M2. An explanation for the discrepancy even after taking metabolism and fetal plasma concentration into account could be bioavailability. It is possible that protein binding of the compounds differs between cell culture media and blood plasma, thus limiting the amount of compound available to bind AR *in vivo*. Another explanation could be that other signalling pathways, not modelled in the *in vitro* setup are involved in modulating the AGD outcome *in vivo*.

Our transcriptional analysis of the male fetuses exposed to enzalutamide *in utero* showed a feminized perineal expression of *Sfrp4* and there was a tendency towards a feminized effect on *Esr1* and *Padi2*, although this was not statistically significant due to large biological variations and a small dynamic range. In our previous study, finasteride exposed males showed a feminized perineal expression of *Esr1* and *Padi2,* but not of *Wnt2* and *Sfrp4* (Schwartz et al, 2019b). In addition, the effects observed following finasteride exposure were more marked suggesting that a more marked feminization of AGD is necessary to detect transcriptional changes in the perineum. In agreement, the short male AGD induced by vinclozolin or procymidone exposure was not associated with any statistically significant changes in perineal gene expression. In general it is important to note that the difference in expression of these four genes between control males and control females was very small, in both the enzalutamide study and the vinclozolin and procymidone study. This limits the dynamic range in which we can expect the gene expression data for the exposed feminized males to be. So even though the mean expression of the four genes for the exposed males in both studies was consistently found between the control males and the control females, statistical significant differences will be very difficult to obtain. According to gene array analysis at GD21, these four genes are, however, the most likely candidates to play a role in the sex specific development of the perineum (Schwartz et al, 2019b). In support, polymorphisms in the human ESR1 is associated with short AGD in male infants (Sathyanarayana et al, 2012). Polymorphisms in ESR1 are also associated with cryptorchidism, hypospadias and low fertility in humans (Ban et al, 2008; Safarinejad et al, 2010; Watanabe et al, 2007), all of which are thought to be associated with short AGD (Skakkebaek et al, 2001). There is less direct evidence that *Wnt2* is associated with short

AGD. It is clear, however, that Wnt plays a role in several androgen dependent processes. Global knock-out of *Ar* affects expression of both *Wnt4* and the Wnt receptor *Fzd4* in male gastrocnemius muscle (MacLean et al, 2008). Exposure to the AR antagonist flutamide reduces protein expression of the Wnt related factor, AXIN2, in the gubernaculum (Chen et al, 2011). Indeed, *Wnt5a* is important for male sexual development including testis descent (Chawengsaksophak et al, 2012).

We performed *ex vivo* cultures of the perineal tissues in an attempt to investigate the mechanism of action of the compounds in more detail, as well as to reduce the variation between the samples. The experimental protocol was adopted from the Pask laboratory, Melbourne, Australia, and has previously been used to grow the genital tubercle and associated perineal tissues from mice in *ex vivo* culture (Govers et al, 2019). While tissues grew well in the 72h *ex vivo* culture, we were not able to reproduce the sexually dimorphic expression of *Esr1* and *Wnt2* that we have consistently seen *in vivo* at GD21. In order to expose the perineal tissues to anti-androgenic compounds within the MPW (van den Driesche et al, 2017; van den Driesche et al, 2011; Welsh et al, 2008), the tissues used for the 72h *ex vivo* culture were isolated from GD15 fetuses. As a result, our gene expression analyses of the *ex vivo* cultures is performed at a developmental stage corresponding to GD18. Based on our preliminary analysis of the temporal expression of *Wnt2* and *Esr1*, the expression of *Wnt2 and Esr1* is similar in males and females at GD18. Changing the timing of the *ex vivo* culture in order to perform gene expression analyses at GD21 would require the tissues to be harvested at GD18. Based on the timing of the MPW, it is unlikely that short AGD can be modelled *ex vivo* by exposure to anti-androgens from GD18-GD21. Another possibility could be to extend the culture period beyond the 72h, albeit with a tissue of this size, it might be difficult to ensure viability of the tissue. An alternative could be to look for other markers that are sexually dimorphic GD18. To this end, transcriptional analysis of the perineal tissues at different stages of development could be of great value. As tissue heterogeneity is a great limitation to the studies of the perineal tissues, single cell sequencing might be a valuable tool in that venture.

Conclusion

We confirm that enzalutamide can be used as a model compound for the effects of AR antagonistic activity on AGD outcome. The discrepancies between the *in vitro* and *in vivo* anti-androgenicity of vinclozolin and procymidone points to important differences in the *in vitro* to *in vivo* extrapolation. As factors such as absorption, metabolism and excretion have been accounted for we speculate that the discrepancies can be caused by differences in

bioavailability or alternative mechanisms of action, which the *in vitro* model cannot account for. There were indications that expression of Wnt and estrogen signaling related factors may be affected in the perineum of males exposed to all of the tested chemicals. Albeit, for vinclozolin and procymidone this could not be proven by statistical methods due to a low power and/or large biological variations. Based on these limited effects, the signaling by the Wnt and estrogen pathways are not likely to explain the discrepancies between the *in vitro* and *in vivo* anti-androgenicity of vinclozolin and procymidone.

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Declaration of interests

The authors declare that they have no conflict of interest concerning this work.

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6

Manuscript IV

6.1 Manuscript IV

Molecular markers of the sexually dimorphic perineum

Schwartz C. L., Svingen T. and Vinggaard A. M. Molecular markers of the sexually dimorphic perineum. *Study report*

Molecular markers of the sexually dimorphic perineum

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Study report

Abstract

Androgen action during fetal life directs the masculinization of many different tissues including skeletal muscle. The levator ani and bulbocavernosus (LABC) muscles of the perineum are especially sensitive to androgen action during fetal life and as a result, they are larger in males than in females at birth. It is unclear, however, if this sexual dimorphism is due to differences in cell proliferation, apoptosis, differentiation or any combination thereof and how androgen action might affect any of these processes. In the present study, we investigated the perineal gene expression of different cell type markers and markers of apoptosis and proliferation. Male and female fetuses were collected by cesarean section from pregnant Sprague-Dawley rats at gestational day (GD)15, GD17, GD18, GD20 and GD21. The perineal tissues were isolated from the fetuses and analyzed by transcriptional and histological analysis. We observed a male-specific increase in the expression of non-myocytic cell marker *Sall1* at GD17 and indications of a male-specific increase in the expression of the myocytic cell marker *MyoG* at GD20-21. These results may support previous literature suggesting that the sexual dimorphism of the perineum stems from a male-specific increase in non-myocytic cells thus providing a larger pool of cells that later differentiate towards muscle specific cell types and result in a larger male LABC. As these results are preliminary, they should be followed up by experiments using sophisticated experimental techniques suitable to answer a question of this complexity.

Introduction

Androgen signaling plays an essential role in masculinization of the male fetus. Androgens direct the development of the male accessory sex organs as well as the sex-specific differentiation of non-reproductive tissues such as skeletal muscles (MacLean & Handelsman, 2009).

The muscles of the perineum, levator ani and bulbocavernosus (LABC), are especially sensitive to androgen action (MacLean et al, 2008; Rand & Breedlove, 1992). While the primordial tissue for these muscles are present in both males and females, it is the high androgen levels in the male fetus that induce LABC to proliferate and differentiate (Cihak et al, 1970; Ipulan et al, 2014). The resulting sex-specific growth of the LABC is thus thought to affect the distance from the anus to the genitals, so called anogenital distance (AGD), which, at birth, is approximately twice as long in males compared to females (Hotchkiss & Vandenbergh, 2005; Salazar-Martinez et al, 2004). As AGD provides a read-out of fetal androgen action, the measurement of AGD is used in toxicological studies to assess the endocrine disrupting potential of chemicals (Schwartz et al, 2019a). Here, a short male AGD is considered a marker of disrupted fetal androgen signaling which is associated with adverse effects on male reproductive health such as genital malformations at birth and late life effects such as low sperm quality, infertility, and testicular germ cell tumors (Dean & Sharpe, 2013; Moreno-Mendoza et al, 2020).

Although most endocrine disrupting chemicals (EDCs) with an anti-androgenic mode of action are thought to affect AGD by blocking androgen receptor (AR) action, the exact molecular mechanism responsible for stunted perineal growth is not well characterized. There is still an ongoing debate as to which cell types within the muscle express AR (Chambon et al, 2010; Ipulan et al, 2014; Ophoff et al, 2009) and which cellular pathways are responsible for the androgen dependent difference in LABC size between males and females. To complicate matters even further, chemicals believed to have an estrogenic or non-AR-mediated mode of action have also been found to affect AGD, suggesting that effects might not only be mediated through AR (Boberg et al, 2016; Christiansen et al, 2014; Holm et al, 2015; Kristensen et al, 2011; Zhang et al, 2014). In this study, we set out to characterize the normal, sexually dimorphic development of the perineum as this could add to our understanding of how EDCs affect AGD by illuminating what other morphoregulatory factors are expressed in the appropriate tissues at the appropriate time.

Methods

Animal study

Nulliparous, young adult Sprague Dawley rats Crl:CD(SD), (Charles River Laboratories, Sulzfeld, Germany) with a body weight of 255±25 g were supplied at gestational day (GD)3. The day of vaginal plug detection was designated GD1. The animals were housed under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden, Lynge, Denmark). They were fed ad libitum standard diet with Altromin 1314 (soy- and alfalfa-free, Altromin GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast). They were kept in an animal room with controlled environmental conditions: 12 hr light-dark cycles with light starting at 9 pm, temperature 22 ± 1°C, humidity 55 ± 5%, 10 air changes per hr.

Caesarean sections

Dams were decapitated under CO_2/O_2-anesthesia at GD15, 17, 18, or 20, and fetuses were collected by caesarean section. Perineal tissues were isolated by dissection under a stereomicroscope and placed in RNAlater for RT-qPCR analysis (Qiagen, Hilden, Germany). Samples were stored at -80 °C until RNA extraction. For histological analyses, whole embryos were placed in formalin. To minimize the use of experimental animals, additional GD21 perineal tissues were obtained from control animals from previous animal studies using the same protocol for housing and caesarean sections (Schwartz et al, 2019b). The animal experiments were performed with ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) and by the in-house Animal Welfare committee.

RNA extraction

Perineal tissues were transferred to 2ml Eppendorf safe-lock tubes containing 350µl (<20mg) or 500µl (>20mg) RLT buffer (Qiagen, Hilden, Germany) and 1% β-mercaptoethanol as well as one 55mm stainless steel ball (Qiagen, Hilden, Germany). The tissue was then homogenized using the TissueLyser II system (Qiagen, Hilden, Germany). Total RNA was extracted from the homogenate using RNeasy mini kits according to manufacturer's instructions (Qiagen, Hilden, Germany).

Synthesis of cDNA and RT-qPCR analysis

To synthesize cDNA 500 ng total RNA was used as well as random primer mix (New England Biolabs, Ipswich, MA, USA) and the Omniscript kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantitative RT-PCR (RT-qPCR) reactions were run in technical duplicates on an QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Thermo Fischer Scientific) in 11µl reactions containing: 3µl diluted (1:20) cDNA, TaqMan Fast Universal Mastermix (2X) (Life Technologies, Carlsbad, CA, USA) and TaqMan Gene Expressions Assays (Life Technologies). TaqMan assays were: *Ar* (Androgen receptor, Rn00560747_m1), *MyoG* (*MyoG*enin, Rn00567418_m1), *Sall1* (Spalt-like transcription factor 1, Rn04244404_m1), *p21* (cyclin dependent kinase inhibitor 1a, Rn00589996_m1) and *Bax* (Bcl2-associated X protein, Rn01480161_g1). The following cycling conditions were used: 95°C for 20 sec followed by 45 two-step thermal cycles of 95°C for 1 sec and 60°C for 20 sec. The relative transcript abundance was calculated using the $2^{-\Delta CT}$ method using *ActB* (β-Actin, Rn00667869_m1) and *Hprt1* (Hypoxanthine guanine phosphoribosyl transferase, Rn01527840) as normalizing genes. Unpaired Student t-test was used to determine statistical significance using the statistical software GraphPad Prism 8 (GraphPad Software, San Diego California, USA). For data presentation, mean ± SEM was calculated from n =3 pups/group.

Histology & immunofluorescence

Whole embryos were fixed in 10% formalin and sectioned at 5µm for histological analysis. For hematoxylin and eosin staining, standard protocols were followed. For immunofluorescent (IF) staining, the 5µm sections were deparafinized in petroleum 2 x 10min, and then rehydrated in 99%, 96%, 70% ethanol and running tap water, each for 5min. Antigen retrieval was carried out in Tris EDTA (pH 9) buffer by heating to 99°C in microwave for 15min. The slides were then cooled for 15min at RT before rinsing in running tap water for 2min and in PBS 3 x 5min. After blocking with 5% BSA for 1h, the slides were incubated with primary antibody overnight at 4°C. The following primary antibodies were used: *SALL1* (Abam, ab31526), *MYOG* (Abcam, ab1835), *MYOD* (Santa cruz, 32758) and *AR* (Abcam, ab74272). The slides were then rinsed in PBS 3 x 5min and incubated with secondary antibody for 1h in the dark. The following secondary antibodies were used: TxRed-568 (Molecular Probes cat# A10042, Lot #1757124), AlexaFluor 488 (ThermoFisher cat# A32723) and GFP-488 (Molecular Probes cat# R37144, Lot #1772706). After rinsing again in PBS 3 x 5min the slides were counterstained with DAPI for 3min and rinsed in PBS 3 x 5min. Lastly, the slides were rinsed in distilled water for 2min before mounting with ProLong Gold Antifade Mountant (ThermoFischer Scientific).

Results

We investigated the expression of markers previously suggested to play a role in the development of the perineal muscles (Ipulan et al, 2014; Jacob et al, 2008). There was a peak in *Sall1* expression in the males at GD17 (Figure 1A). *MyoG* expression steadily increased between GD15 and GD18 in both males and females although this was not statistically significant (Figure 1B). In females, the *MyoG* expression plateaued from GD18 to GD21 whereas it seemingly, but not statistically significant, continued to increase in the males (Figure 1B). The expression of *Ar* in the perineum peaked in the females at GD18, and here expression was higher in females than in males (Figure 1C). In males, there was a peak in expression of *Ar* at GD17 and GD21. The pattern of temporal expression of *Bax* was similar to that observed for *Ar* (Figure 1D). There were peaks in expression of *Bax* in males at GD17 and GD21. At GD18 *Bax* expression was higher in females than males and at GD21 expression was higher in males than in females (Figure 1D). The expression of *p21* increased over time in both males and females (Figure 1E).

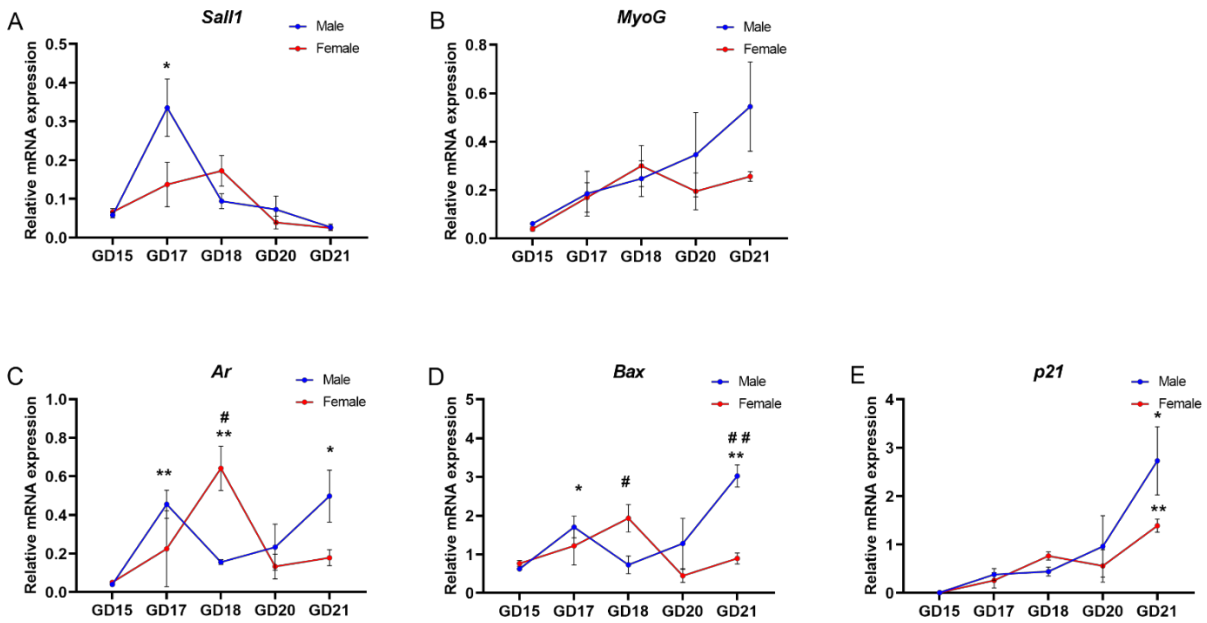

Figure 1. Temporal gene expression in male and female. **A**: Expression of *Sall1* showed a male-specific peak at GD17. **B**: *MyoG* expression seemed to gradually increase over time although this was not statistically significant. **C**: There were peaks in the expression of *Ar* at GD17 and GD21 in males. In females, *Ar* expression peaked at GD18 where it was higher in females compared to males. **D**: *Bax* expression peaked in the males at GD17 and GD21 and was higher at GD21 in males compared to females. At GD18 expression was higher in females compared to males. **E**: Expression of *p21* increased in both sexes over time. Results are $2^{-\Delta CT}$ values shown as mean ± SEM and n=3 fetuses/group. GD: Gestation day. Blue: male. Red: female. *: Significantly

different from GD15 ($p < 0.05$). **: Significantly different from GD15 ($p < 0.01$). #: Significantly different from the other sex at the given time point ($p < 0.05$). ##: Significantly different from the other sex at the given time point ($p < 0.01$).

Histological examination of the male fetus at GD17 was used to locate the perineum (Figure 2A). To determine the relative contribution of non-myocytic cells and muscle cells to the perineum we performed immunofluorescence (IF) staining for the non-myocytic marker SALL1 and the muscle cell markers MyoD and MyoG on whole embryo sections. Expression of SALL1 could be detected specifically in the perineal area (Figure 2B) while IF staining for MyoG and MyoD did not work (data not shown). We found that AR was expressed in the male perineum at GD17 (Figure 2C).

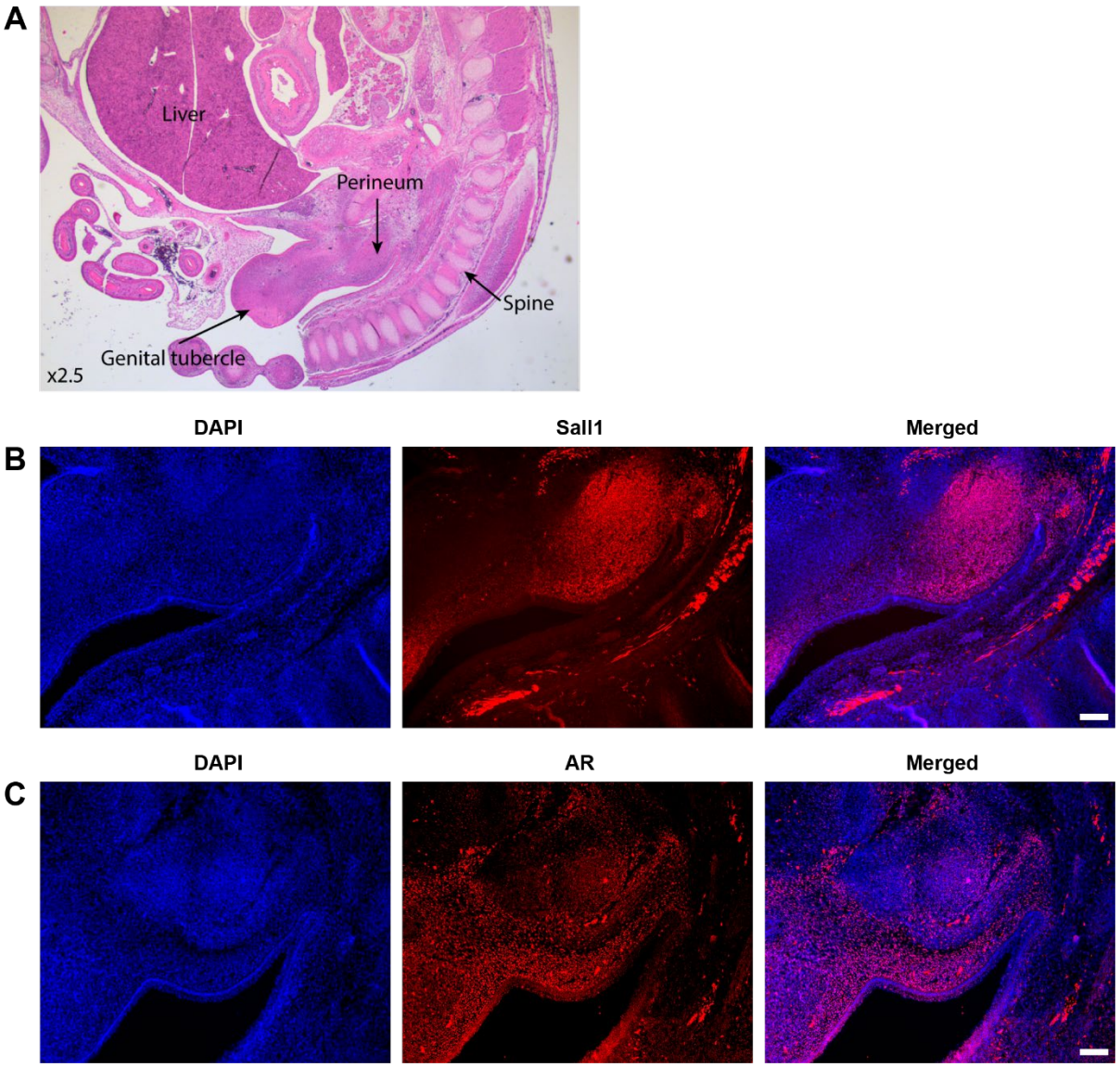

Figure 2. Histological assessment of saggital sections of the perineum from male fetus at GD17. **A:** H&E staining. The perineum, genital tubercle, spine and liver are indicated for orientation. **B:** Localization of *SALL1* expression (red) in the perineum and nuclear counterstain with DAPI (blue). **C:** Localization of *AR* expression (red) in the perineum and nuclear counterstain with DAPI (blue). Scale bar = 100 μm.

Discussion

Several studies show that the muscles of the perineum are highly sensitive to androgen action (Cihak et al, 1970; Ipulan et al, 2014; Rand & Breedlove, 1992; Wainman & Shipounoff, 1941), which in part explains why AGD is directly related to fetal androgen levels. It is still debated, however, which cell types drive the androgen mediated effects on the perineum (Chambon et al, 2010; Ipulan et al, 2014; Ophoff et al, 2009), and whether ultimate size difference of the LABC is due to differences in cell differentiation, proliferation, apoptosis or a combination thereof.

The male-specific increase in the expression of the non-myocytic cell marker *Sall1* at GD17 suggests that the pool of non-myocytic cells only increases in the males. This peak in Sall1 expression coinsides with an increase in Ar expression also in the males. Whereas expression of the muscle cell marker *MyoG* plateaued in females at GD18 it seemingly continued to increase in males from GD18 to GD21 although the effect was not statistically significant, possibly due to the high variation. This data may indicate that the high androgen levels in male fetuses induce proliferation of *Sall1* cells, thereby providing more precursors for the subsequent myogenic process. This would ultimately lead to are larger LABC muscle complex in males compared to females. Indeed, previous studies indicate that AR signaling induces proliferation of the myogenic precursor cells (Ipulan et al, 2014). Expression of the cell cycle regulating gene *Cdkn1c,* a cyclin dependent kinase inhibitor (Cdk), is increased in the muscle of mice with global knockout of AR (MacLean et al, 2008). Together with another Cdk, *p21*, these have been shown to play a vital role in skeletal muscle differentiation by triggering cell cycle exit and myoblast differentiation (Zhang et al, 1999). Supporting a role for this process in the perineum, *p21* expression is increased in the BC region following AR knockout specifically in *Sall1* positive cells (Ipulan et al, 2014). Together this suggests that AR promotes proliferation in non-myocytic, *Sall1* positive cells by inhibiting cell cycle exit. Based on this, expression of *p21* would be expected to be higher in females than in males in the present study. However, we did not observe any differences in *p21* until GD21 and here there was an indication towards a higher expression in males compared to females. It is possible that other Cdks are responsible for the observed effects, a

difference that could be due to e.g. the choice of species as the rat was used in the present study whereas mice have been used in the previous studies.

While our preliminary gene expression data may corroborate previous findings of cell-specific expression of above-mentioned factors, this expression pattern is not proof that this cell type is directly contributing to the LABC complex; the data are only indicative. Two previous studies suggest that the androgen mediated growth of the LABC depends on AR expression in myocytic cells (Chambon et al, 2010; Ophoff et al, 2009). By contrast, another study finds that the androgen driven effects on LABC are mediated by AR expressed in non-myocytic, *SALL1* positive cells and not in *MYOG* positive cells (Ipulan et al, 2014). Using immunofluorescence we wanted to confirm the relative contribution of the non-myocytic and myocytic cells to the perineum at the different stages of development and investigate the cell type specific AR expression. While we could confirm the presence of *SALL1* and *AR* positive cells in the male perineum at GD17, staining of the myocyte markers *MYOG* and *MYOD* did not work. At present we have therefore not been able to confirm our results.

Simultaneous ablation of the two pro-apoptotic genes *Bax* and *Bak* greatly increases the LABC size in females (Jacob et al, 2008), suggesting that their expression is key to the sexually dimorphic development of the LABC. In female perineum, we observed an increase in the expression of *Bax* at GD18 that coincides with a peak in *AR* expression. A previous study that showed that AR signaling promoted Bax-mediated apoptosis in prostate cancer cells (Lin et al, 2006). This could suggest that AR signaling in the female induces Bax-mediated apoptosis of the non-myocytic cells. This would imply, however, that the effect of AR signaling is different in *SALL1* positive cells dependent on the sex. While the chromosomal sex of the cell can dictate its response (Arnold & Chen, 2009; Du et al, 2004; Penaloza et al, 2009), it seems unlikely that this is the case in the present study. If so, disrupted AR signaling should make the female AGD longer. By contrast, several studies have measured female AGD following *in utero* exposure to anti-androgenic chemicals and found no effect on AGD (Schwartz et al, 2019a). In the present study we also observed an increase in *Bax* expression in males over time and *Bax* expression was higher in males than in females at GD21. This suggests that apoptosis also takes place in the male perineum. Indeed, baseline cell turnover is suggested to be a normal part of muscle development (Jacob et al, 2008). Thus, more work is needed to characterize the regulation of apoptosis in the LABC during development and elucidate how this contributes to the sexual dimorphism of this muscle complex.

The samples investigated in this study are very heterogeneous, and changing over time. To confirm the findings of this preliminary study and to better depict the complexity of these

developmental processes, more sophisticated techniques should be applied. Methods such as bulk RNA barcoding (BRB) sequencing (Alpern et al, 2019) would allow us to get detailed transcriptional information while vastly increasing the sample size for each time point of development, thus increasing statistical power and reliability of the results. To better track cells through their developmental trajectories over time, single cell sequencing could be a valuable option, especially to determine the relative contribution of different cell type population to the perineal tissues. However, it does not provide any information on the spatial organization of the identified cell types. In the present study, we have used IF to get information of the spatial organization based on protein expression. A drawback to this method is the dependence on the availability and quality of antibodies. The *in situ* hybridization technique RNAscope (Wang et al, 2012) uses a unique probe design to attain single cell RNA expression information in a morphological context. Combining the high throughput of single cell sequencing with the spatial information from RNAscope seems like an intriguing way to characterize the sex-specific development of the perineal tissues.

Conclusion

The present preliminary data might support previous literature suggesting that proliferation of the non-myogenic cell population specifically in the males provides a greater pool of precursors for myogenic differentiation and thus forms the basis for the sexually dimorphic size of the LABC muscles of the perineum.

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7

General discussion

7.1 Overview of the main findings

Below is a short summary of the aim, methods and main findings from each of the manuscripts, which are then discussed in a broader context in the subsequent sections.

Chapter 3 – Manuscript I

Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders

The aim of this literature study was to collate the available knowledge related to *in utero* xenobiotic exposure and AGD measurements. The review presents a discussion on the utility of AGD as a retrospective biomarker of fetal androgenicity in human epidemiology and in rodent toxicity studies. Data from more than 100 rodent toxicity studies, covering close to 50 chemical compounds, were included to discuss how different chemical classes might affect AGD. The review highlights four main questions that need to be addressed in the future to fully evaluate the utility of AGD as a biomarker; i) Are other morphoregulatory pathways than androgen signaling involved in determining the AGD outcome? ii) Is there a linear relationship between the magnitude of shortening in AGD and the risk for reproductive disorders? iii) To what extent can AGD be used as a stand-alone biomarker of anti-androgenic effects? iv) Can AGD measurements be used as a biomarker in females?

Chapter 4 – Manuscript II

Distinct transcriptional profiles of the female, male and finasteride-induced feminized male anogenital region in rat fetuses

The aim of this study was to characterize the molecular mechanisms affected in males with finasteride-induced, short male AGD.

To induce short AGD in the male offspring, we exposed pregnant Sprague-Dawley rats to finasteride (10mg/kg bw/day) by oral gavage from GD7-GD21. At GD21, fetuses were collected and AGD measured. The perineal tissues were isolated for gene array analysis and RT-qPCR.

The gene array analysis of the perineal tissues revealed that the control males, control females and exposed males had distinct transcriptional profiles. There was a closer relationship in the perineal transcriptional profile between exposed males and control females than between exposed males and control males, suggesting a feminization of the perineum of exposed males. Four genes related to Wnt and estrogen signaling (*Wnt2, Sfrp4, Esr1,* and *Padi2*) had a sexually dimorphic expression pattern.

As expected, finasteride induced short AGD in the exposed male fetuses and induced effects on the perineal gene expression of the estrogen related factors *Esr1* and *Padi2.* In conclusion, a short, feminized male AGD is associated with a feminized transcriptional profile of the perineal tissues. Furthermore, the Wnt and estrogen signaling pathways might play a role in the sex-specific development of the perineal tissues and in the 'feminization' of perineum in males with chemically induced short AGD.

Chapter 5 – Manuscript III

Expression of estrogen and Wnt related factors in the rat perineal tissues during normal development and following disruption of fetal androgen action

The aim of this study was to investigate effects on expression of *Wnt2, Sfrp4, Esr1,* and *Padi2* in males with short AGD following *in utero* exposure to chemicals with an AR-antagonistic mode of action.

Pregnant Sprague-Dawley rats were exposed to enzalutamide (10mg/kg bw/day), vinclozolin (40mg/kg bw/day), or procymidone (40mg/kg bw/day) from GD7-GD21. At GD21, the chemical exposure concentrations were measured in amniotic fluid and fetal plasma, AGD was measured, and the perineal tissues were isolated for gene expression analysis of the four genes *Wnt2, Sfrp4, Esr1,* and *Padi2*. To obtain a timeline for the expression of these four genes, non-dosed male and female fetuses were investigated at GD15, -17, -18, -20 and -21. In addition, the anogenital region was isolated from non-dosed rat offspring at GD15 and cultured *ex vivo* for 72h in culture media containing either enzalutamide (1µM or 100 µM) or vinclozolin (1µM or 100µM).

The temporal expression analysis of *Wnt2, Sfrp4, Esr1,* and *Padi2* showed that expression of these factors was similar in males and females at least until GD17. *In utero* exposure to enzalutamide induced short AGD in male fetuses and affected perineal gene expression of the Wnt related factor *Sfrp4*. Vinclozolin and procymidone also induced short AGD in exposed male fetuses but did not significantly affect the perineal gene expression of *Wnt2, Sfrp4, Esr1,* or *Padi2*. For all three chemicals there were indications of effects on all of the four genes tested, but due to low power and high variation this could not be statistically proven. We could not reproduce the sexually dimorphic expression of *Esr1* and *Wnt2,* that we have consistently seen *in vivo,* in the perineal *ex vivo* cultures. This setup therefore needs more work before it can be used to model short AGD.

Chapter 6 – Manuscript IV

Molecular markers of the sexually dimorphic perineum

The aim of this preliminary study was to investigate the normal, sexually dimorphic development of the perineum.

Male and female fetuses from non-dosed pregnant Sprague-Dawley rats were collected at GD15, -17, -18, -20 and -21. The perineal tissues were isolated for gene expression analysis and histological analysis of cell type markers and markers of apoptosis and proliferation.

Gene expression analysis of *Sall1* and *MyoG* suggests a male-specific increase in non-myocytic progenitor cells in the perineum during early development and that these cells provide the basis for myogenic differentiation at later stages of fetal development. Immunofluorescent staining confirmed the expression of SALL1 and AR in the perineum. These are very preliminary data and should be followed up by larger, more detailed studies using techniques suited for tracking cell populations in a spatiotemporal context.

7.2 Molecular mechanisms of short male anogenital distance

Two main questions need to be addressed to fully answer the hypothesis that the effects of EDCs on male AGD are not solely due to disruption of androgen signaling. The first question is, is there cross-talk between androgen signaling and other signaling pathways that may affect AGD outcome following *in utero* exposure to anti-androgens? The second questions is, are there other signaling pathways that, instead of or in parallel to androgen signaling, that can explain the effects seen on AGD following exposure to chemicals? The second question has not been directly addressed in this PhD project, as time constraints did not make it possible to set up more *in utero* exposure experiments. This is, however, an important point to address and will be discussed further in section 7.8 *Perspectives*. But first, question one is addressed.

The identification of sexually dimorphic expression of Wnt and estrogen related factors during perineal growth in rats (chapter 4), and the finding that these pathways were dysregulated in incompletely masculinized ('feminized') males (chapter 4 and 5), suggest that they interact with androgen signaling to induce effects on AGD. In fact, it is already known that estrogen receptors are expressed in the genital tubercle during development and beyond in both rodents (Agras et al, 2007; Goyal et al, 2004; Jesmin et al, 2002) and humans (Crescioli et al, 2003; Dietrich et al, 2004) and that the androgen-estrogen balance may be involved in phallus differentiation. In particular, this hormonal relationship has been highlighted as a potential mechanism for hypospadias formation, as both estrogen deficiency and excess can induce this reproductive-tract malformation (Agras et al, 2007; Fernandez et al, 2016; Govers et al, 2019; Stewart et al, 2018). What remains unknown, however, is if this

androgen-estrogen balance is also important in the perineal tissues and can contribute to changes in AGD.

In support of a role for estrogen in the programming of AGD, polymorphisms in ESR1 are associated with short male AGD in humans (Sathyanarayana et al, 2012). In addition, *in utero* exposure to chemicals with an assumed estrogenic mode of action such as butyl paraben (Byford et al, 2002; Routledge et al, 1998) and bisphenol A (Kim et al, 2001; Matthews et al, 2001) can, in some studies, induce short male AGD (Boberg et al, 2016; Christiansen et al, 2014; Zhang et al, 2014). Together with previous studies (Ipulan et al, 2014; Jacob et al, 2008), the results in chapter 6 suggest that the sexually dimorphic development of the perineum hinges on a balance between apoptosis, proliferation, and myogenic differentiation of progenitor cells. It is possible that estrogen action in the muscle can affect these processes. Estrogen binding has been detected in the LABC (Dionne et al, 1979). In ovariectomized female mice, treatment with estradiol valerate reduced skeletal muscle mass (Ogawa et al, 2011). In agreement, 17β-estradiol and BPA reduce myogenic differentiation of murine C2C12 myoblasts *in vitro* (Go et al, 2018; Ogawa et al, 2011). By contrast, exposure to the phytoestrogen formononetin increases myoblast differentiation (Soundharrajan et al, 2019). However, the finding that perineal expression of *Esr1* is higher in finasteride-exposed males with short AGD (chapter 4) and in control females (chapter 5) compared to control males, might support that estrogen represses myogenic differentiation. Based on these observations one might speculate if estrogen acts through *ESR1* to repress myogenic growth and differentiation in the LABC of females and feminized males and thereby contribute the shorter AGD.

To my knowledge, there is no previous literature linking Wnt signaling to AGD. There is, however, literature that might support a role for Wnt in the proliferation and differentiation of cells in the male perineum. Wnt signaling is known to be involved in embryonic muscle determination and to interact with AR (Cossu & Borello, 1999; Mulholland et al, 2005; Tran et al, 2013). Wnt induces cell proliferation in many different contexts and inhibition of Wnt signaling has been shown to reduce proliferation of C2C12 myoblasts *in vitro* (Suzuki et al, 2015). In addition, global ablation of AR in mice affects expression of *Wnt4* and the Wnt signaling receptor *Frizzled 4* (Fzd4) in the gastrocnemius muscle. This might support that Wnt signaling could be involved in AR mediated effects on LABC muscle development and AGD. The Wnt antagonist *Sfrp4* is a pro-apoptotic factor (Melkonyan et al, 1997) and regulates apoptosis and proliferation in, for example, ovulation, mammary gland involution and prostate cancer development (Drake et al, 2003; Horvath et al, 2007; Lacher et al, 2003). As shown in chapter 4, *Wnt2* expression is lower in females than in males at GD21 whereas

Sfrp4 is higher in females than in males. The same pattern is seen at certain time points in the timeline data from chapter 5. This could suggest that the high *Wnt2*-low *Sfrp4* expression in males induces proliferation of progenitor cells in the male perineum while the low *Wnt2*-high *Sfrp4* expression in females induces apoptosis of cells in the female perineum. This would be in line with previous data suggesting that the near absence of LABC in females is due to apoptosis (Cihak et al, 1970; Jacob et al, 2008).

Collectively, there are data to support a role for Wnt and estrogen signaling in cell proliferation and differentiation in the fetal LABC in rodents. It is important to note, however, that the effects observed on Wnt and estrogen presented in chapter 4 and 5 were not always consistent and that the timeline data presented in chapter 5 and 6 are very preliminary. In order to draw any reliable conclusions, much further research is required to elucidate the role of these pathways in the development of LABC.

7.2.1 Fetal programming of adult AGD

To the best of our knowledge, the AGD is largely programmed by androgen action during the MPW (van den Driesche et al, 2017; Welsh et al, 2008) and is considered to be relatively stable throughout life. There is, however, evidence that AGD is partially responsive to changes in the androgen-estrogen balance in postnatal life (Kita et al, 2016; Mitchell et al, 2015). Differences in how androgens mediate effects on muscle growth in fetal and adult life might explain how AGD can be modulated postnatally. In adult male rats, LABC size decreases following castration and increases following testosterone treatment (Venable, 1966; Wainman & Shipounoff, 1941). This is a result of changes in muscle fiber *size* rather than in muscle fiber *number* (Venable, 1966). In fetal male rats, however, AR activity increases cell proliferation in the LABC (Ipulan et al, 2014). The results in chapter 4-6, as discussed above, may also support that fetal androgen action induces proliferation in male perineum. This indicates that androgen action during fetal life programs AGD by defining the final number of muscle cells within the LABC while androgen action during postnatal life can slightly modulate AGD by inducing growth of the existing muscle cells.

7.3 Molecular knowledge in a regulatory context

With the current focus on reducing animal studies and relying more on alternative, non-animal test methods, there is a concomitant need for detailed mechanistic knowledge of relevant toxicological pathways. Therefore, in addition to asking *if* EDCs can affect reproductive health, it is important that we also ask *how*. The aim of the present PhD project was to identify potentially novel signaling pathways affected in the perineal tissues of males with short AGD. The hypothesis was that the effects of EDCs on male AGD are not

solely due to disruption of androgen signaling, but also includes other morphoregulatory pathways. Although further research is warranted, my results suggest that Wnt and estrogen signaling in the perineum could be additional pathways involved in the effect on AGD. As discussed below, this could suggest that Wnt and estrogen modules may be incorporated into AOPs for effects on AGD.

7.3.1 AOP development

In the AOP framework, existing knowledge of toxicological pathways is organized into a series of events leading from molecular initiating event through causally linked key events to an adverse outcome. Important to the AOP principle is that the AOPs are dynamic; they can be thought of living documents that can be elaborated upon as the mechanistic knowledge of toxicological pathways increases (Villeneuve et al, 2014). In the present studies, the Wnt and estrogen signaling pathways were affected in males with short male AGD (chapter 4 and 5). As they were affected in response to anti-androgenic treatment this suggest that they partake in cross-talk with the androgen signaling pathway and should therefore be incorporated into *existing* AOPs for short male AGD (Figure 1). Although not examined in the present project, it is also possible that Wnt and estrogen can act independently of androgen signaling to affect AGD. In this case, factors of the Wnt and estrogen signaling pathways may be molecular initiating events for new, individual AOPs (Figure 1). Future studies using chemicals with a non-AR mediated mode of action are needed to answer this as discussed further in section 7.8 *Perspectives*.

As previously described, only events that are key to the progression of the pathway towards an adverse outcome are to be included in the AOP. Based on the relatively small effects observed on the Wnt and estrogen signaling pathways (chapter 4 and 5) they might not play a significant role in determining AGD. In addition, because the effects were observed at GD21, it is possible that Wnt and estrogen signaling are not causally linked to short AGD but rather are an effect of changes at earlier stages of development. Therefore more evidence is needed before a decision can be made on the inclusion of these pathways in the AOPs for short male AGD.

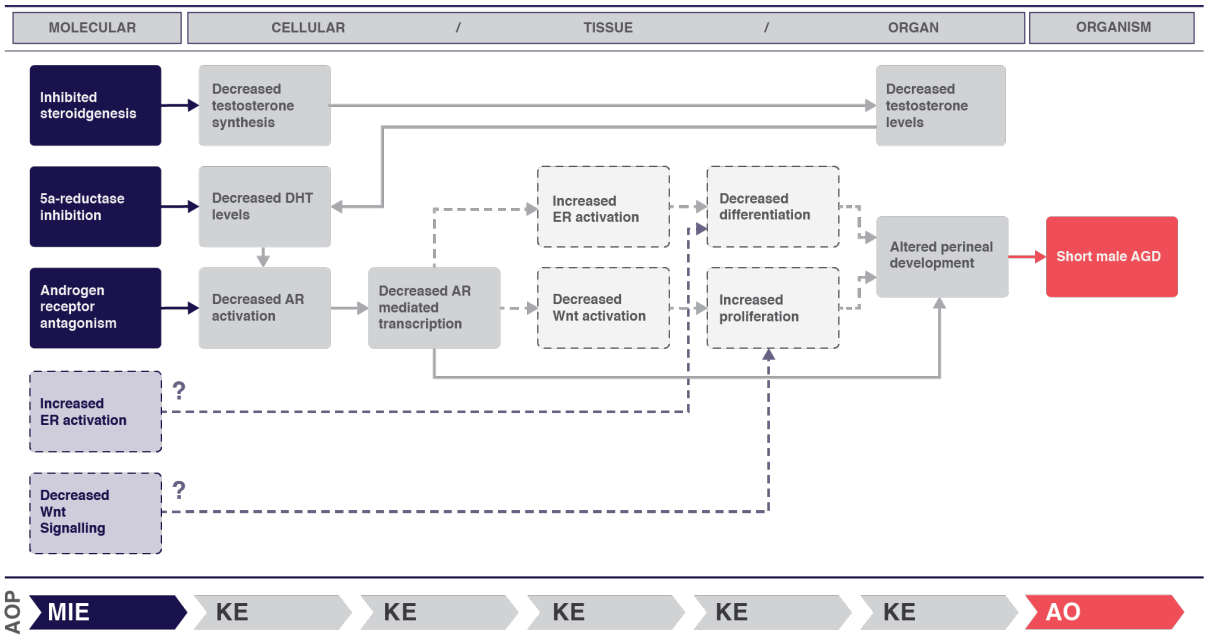

Figure 1. Possible adverse outcome pathway (AOP) network for short male anogenital distance (AGD). Based on the current mechanistic knowledge, three AOPs for short male AGD are under development at AOPwiki.org (AOPwiki, 305; AOPwiki, 306; AOPwiki, 307) originating from three individual molecular initiating events (MIEs; dark blue boxes). Through causally linked key events (KEs; dark grey boxes) they lead to the adverse outcome (AO; red box), which in this case is short male AGD. The results presented in chapter 4-5 suggest that there may be cross-talk between androgen signaling and the Wnt and estrogen signaling pathways. If their relevance is confirmed by future studies they might be incorporated as KEs into the existing AOP network as shown here (light grey boxes with dotted lines). Although not addressed by the studies of this PhD project, it is also possible that Wnt and estrogen signaling can act independent of androgen signaling to induce short male AGD. In this case, Wnt and estrogen signaling might be added as molecular initiating events (light blue boxes and dotted lines).

7.3.2 Wnt signaling as a target for EDCs

As described in section 2.1.3 *Adverse outcome pathways and new approach methodologies*, the OECD has highlighted seven non-EATS modalities for ED (OECD, 2012). It is clear, however, that chemicals might induce ED effects through many other modalities not mentioned in this OECD review paper such as Wnt and Hedgehog (Johansson & Svingen, 2020). Wnt signaling is evolutionarily conserved and plays a vital role for many processes of embryogenesis including body axis patterning, cell fate specification, cell proliferation, and cell migration. An example is the involvement of Wnt in development of the reproductive

organs. Factors of the Wnt4/RSPO1/β-catenin pathway are essential for ovarian differentiation (Nicol & Yao, 2014). Their crucial role in gonadal sex determination is evidenced by the testis-to-ovary reversal induced in both rodents and humans following overexpression of these factors (Jordan et al, 2001; Maatouk et al, 2008). Another factor of the Wnt pathway, *Wnt5a,* is expressed in the fetal testis and gubernaculum in rodents (Chawengsaksophak et al, 2012; Clement et al, 2007) and ablation of *Wnt5a* in mice induce adverse effects such as testicular hypoplasia and bilateral cryptorchidism (Chawengsaksophak et al, 2012). In addition, the present findings (chapter 4 and 5) suggest that *Wnt2* might modulate androgen signaling to affect AGD. In the latter case, one could speculate if Wnt signaling also regulates androgen signaling in other tissues as Wnt is known to interact with the AR in many different contexts as previously reviewed (Mulholland et al, 2005). Collectively, this could suggest that chemicals might cause adverse effects on reproductive health by interfering with Wnt signaling. The effect could then either be direct or possibly mediated by modulation of androgen signaling. If future work confirms that chemicals can induce ED effects by interfering with Wnt signaling, this modality should be included in AOPs and as an endpoint in future chemical testing strategies for regulatory purposes.

7.4 *In vitro* to *in vivo* extrapolation

One focus of this PhD project was to find molecular mechanisms that could possibly lay the foundation for future development of improved testing strategies for chemical risk assessment. The development of such approaches capable of predicting the *in vivo* outcome in humans requires detailed mechanistic knowledge. There are currently *in vitro* assays for several of the molecular events that lead to short male AGD such as the AR Eco assay for AR antagonism (OECD, 2016c) and the H295R assay for steroidogenesis (OECD, 2011). Utilizing results from such *in vitro* assays to predict *in vivo* reproductive toxicity is, however, complicated by the kinetic dynamics of compounds within the living organism (Gray et al, 2020). Some chemicals, e.g. vinclozolin used in the present study, are quickly metabolized to active metabolites *in vivo* while others may be poorly absorbed, distributed only in certain compartments, be metabolically inactivated, or rapidly excreted. NAMs such as quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) methods make use of physiologically based kinetic (PBK) models to simulate such parameters. QIVIVE hold great promise for improving the utilization of *in vitro* data to predict *in vivo* effects and thus minimize unnecessary *in vivo* testing in future. Indeed, a recent QIVIVE approach could, for three pesticides with *in vitro* anti-androgenic activity, predict the fetal levels attained after maternal dosing, as well as their *in vivo* anti-androgenicity measured as shortening of male

AGD (Scholze et al, 2020). The success of such QIVIVE approaches depends on the inclusion of *in vitro* methods that model the most relevant mechanisms for a given effect as well as detailed toxicokinetic information. Thus, to accurately predict AGD outcome it may be relevant to model other mechanistic events in addition to AR antagonism and suppression of androgen synthesis. If future studies confirm that the Wnt and estrogen pathways contribute to the effect on AGD following exposure to different chemicals, and that these mechanisms are also relevant to humans, these modalities should be considered in future testing strategies for endocrine disruption.

7.5 Rodent to human extrapolation

The experimental work of this project relied on the rat as a model species for characterizing molecular changes in the perineum of male offspring displaying a short AGD induced by intrauterine disruption to androgen signaling. The rat is a valuable model species, commonly used in reproductive toxicology. Although epidemiological studies can provide important information regarding exposure levels and disease outcome, the cause-effect relationships can rarely be proven in humans. Assessment of the risk that chemicals pose to human health thus has to rely on model systems based on *in vitro, in silico* and *in vivo* methods to represent humans. A prerequisite for this is, of course, that the modelled mechanistic events and adverse outcomes are relevant to human pathophysiology.

In both humans and rodents, a short male AGD is considered a biomarker for compromised androgen action during the MPW (Schwartz et al, 2019; van den Driesche et al, 2017; Welsh et al, 2008). However, as outlined below and recently reviewed (Sharpe, 2020), there are fundamental differences in the regulation of androgen production between rodents and humans that should be taken into account when evaluating – and most importantly, extrapolating – rodent reproductive toxicity for human risk assessment purposes.

Masculinization of the male rodent fetus during the MPW relies on testicular steroidogenesis for the production of testosterone, then subsequent conversion to DHT at the target tissues by 5α-reductase. While this is also considered the main pathway responsible for masculinization of the human fetus during the MPW, the alternative "backdoor pathway" also plays an essential role in humans (Fluck et al, 2011; O'Shaughnessy et al, 2019). Here, placental progesterone is converted to androsterone in the placenta, liver, adrenals and, to a lesser degree, the fetal testis, providing a substrate for conversion to DHT at the target tissues by the enzymes *ANKR1C2* and *ANKR1C4*, thus circumventing the canonical testicular steroidogenesis. This means that anti-androgenic chemicals which exert their effect by targeting steps of the canonical steroidogenesis pathway may not affect DHT production by the backdoor pathway. For chemical risk assessment, this implies that rodent

toxicological studies of reproductive toxicity may not accurately mimic the same effects in the human, simply because DHT produced by the backdoor pathway may alleviate some of the effects. In chapter 4, we used the 5a-reductase inhibitor finasteride, to induce short male AGD. This, therefore, represents an inhibition of the canonical steroidogenesis pathway with a resulting effect on the estrogen signaling pathway in the perineum. It is conceivable that in the human fetus, DHT derived from placental androsterone would alleviate this effect and thus, an inhibitor for 5a-reductase in rats may not accurately model anti-androgenic effects in the human. The enzyme *AKR1C2*, which converts androsterone to DHT, is expressed in the human genital tubercle (O'Shaughnessy et al, 2019), but it remains to be determined if similar enzymes are expressed in the perineum and thus to what degree this backdoor pathway can affect AGD outcomes. If this is the case, model compounds that target AR, as those used in chapter 5, could be more appropriate when modelling the human system as this is the receptor for DHT regardless of the source.

Another important difference between rodents and humans concerns estrogen signaling within the fetal testis. In rats, *in utero* exposure to the synthetic estrogen DES results in a marked reduction of fetal intra-testicular testosterone levels (Haavisto et al, 2001; Mitchell et al, 2013; van den Driesche et al, 2012). These effects are not relevant to the human system because ESR1, which mediates the effects of estrogen in the fetal rat testis, is not expressed in human fetal testis (Mitchell et al, 2013). This suggests that testicular steroidogenesis in the human fetus is not affected by EDCs acting via ESR1. ESR1 is, however, expressed in the genital tubercle of both humans and rodents, and disruption of normal estrogen signaling can induce hypospadias (Crescioli et al, 2003; Dietrich et al, 2004; Fernandez et al, 2016; Govers et al, 2019). This suggests that while EDCs with an estrogenic mode of action do not affect testosterone production in the fetal testis they can still induce reproductive tract malformations by mechanisms that are relevant to humans. With the identification of a possible role for *Esr1* in masculinization of the perineum in the male rat fetus (chapter 4 and 5), it would be interesting to investigate if ESR1 is expressed in the human perineum.

The above-mentioned points highlight the importance of mechanistic knowledge when extrapolating results between species. This underlines the importance of understanding the molecular mechanisms controlling AGD outcome in the rodent model as a way of assessing which chemically-induced effects on AGD from toxicity studies that are relevant to human pathophysiology.

7.6 Evaluating and reporting AGD data

When searching the available literature pertaining to xenobiotic exposure and AGD (chapter 3), it became apparent that there are many different ways of measuring and reporting AGD. This can affect the ability to compare different studies but also how reliable the reported data are. It is clear that several factors should be considered when reporting and evaluating AGD data. As described in chapter 2, AGD is correlated with body weight, which should be adjusted for, preferably by calculating AGDi or as a co-variate in the statistical analysis (OECD, 2008). The timing of AGD measurement must also be considered when comparing data from different studies. According to the OECD test guidelines 443, 421/422 and 414, AGD measurements are mandatory to perform in all live fetuses/pups at either gestational days 20-21 (as close as possible to the normal day of delivery) or postnatal days 0–4 (OECD, 2016a; OECD, 2016b; OECD, 2018b; OECD, 2018c). A factor that is difficult to account for when reporting AGD data is exactly how the measurement was made. In contrast to measuring for example the length of a limb, the measurement of AGD is difficult because the perineum is elastic. The precise value of the measurement can be affected by how much the fetus is extended and by the exact angle at which the fetus is held while measuring. As the effects of environmental chemicals on AGD following *in utero* exposure may be subtle, precise measurements of AGD in toxicity studies are imperative to provide correct data for use in chemical risk assessment. Table 1 provides an overview of historical data of AGD at GD21 based on control experiments on both Wistar rats and Sprague Dawley rats in our laboratory and a laboratory in France (OECD, 2018a).

Table 1. Historical AGD data

	AGDi mean (mm/g)	AGDi standard deviation (mm/g)	AGDi coefficient of variation (%)	Control experiments (range of litters)
All control experiments	2.00 ± 0.36	0.08 ± 0.04	4.03 ± 1.67	17 (3-25)
Control experiments with Wistar rats	2.31 ± 0.14	0.10 ± 0.04	4.49 ± 1.69	9 (3-18)
Control experiments with Sprague-Dawley rats	1.72 ± 0.21	0.07 ± 0.02	3.99 ± 1.09	8 (6-25)

Overview of male AGD in control animals at GD 21. Male AGD mean ± SD. Averages for all control group means, control group standard deviations (calculated using litter means) and control group coefficients of variation (of litter means) are given. Coefficient of variation is defined as the ratio of the standard deviation to the mean of the sample. In relation to all calculated averages, the standard deviation (of litter means) is depicted. The total number of studies and the range of the number of litters for the control groups are also given. Importantly, the controls are also split with regards to the rat strain used. The data

AGDI mean is a group mean (control) based of litter means in each study. This table and associated text is taken from the feasibility study for minor enhancements of OECD test guideline 414, no. 285 (OECD, 2018a)

This group sizes and the low coefficient of variation (Table 1) gives the statistical power for detecting significant changes in AGD of ~5% (OECD, 2015). Using more advanced statistical models, a statistically significant change in AGD of only 2% have previously been detected in response to *in utero* exposure of a mixture of EDCs (Ramhoj et al, 2018). The biological significance must be considered for such observations. In the same study, using the full statistical model, the 2% reduction in AGD was associated with effects such as reduced the weight of the epididymides, ventral prostate and seminal vesicles (Ramhoj et al, 2018). Other studies using less specific statistical models, report a significant decrease in AGD of 3-5% in exposed animals compared to controls (Hass et al, 2012; Nardelli et al, 2017; Saillenfait et al, 2013; Saillenfait et al, 2017). In some of the studies, this effect on AGD was not associated with any effects on the male reproductive system, while in others it was associated with reduced testis testosterone and reduced expression of steroidogenesis genes (Saillenfait et al, 2017) and an increase in hemorrhagic testes and multinucleated gonocytes (Nardelli et al, 2017). Studies reporting a 10-15% shorter AGD observe effects such as reduced reproductive organ weights, reduced sperm count and dysgenesis of the testis and external genitalia (Christiansen et al, 2010; Hass et al, 2012; Martino-Andrade et al, 2009; McKee et al, 2006; Wolf et al, 1999). However, as highlighted in chapter 3 it remains to be determined if there is a linear relationship between the magnitude of shortening in AGD and the risk for reproductive disorders (Christiansen et al, 2008; Gray et al, 2001; Schwartz et al, 2019).

AGD data are used in a regulatory context to set the "No Adverse Effect Level" (NOAEL) (OECD, 2008; OECD, 2013), highlighting the importance of the reliability of the data. The observations above outline some of the factors that should be considered in the future when reporting and evaluating data on AGD.

7.7 Considerations for the applied methodology

There is limited literature regarding the fetal perineal tissues. While this presents an interesting and important knowledge gap, it led to a very time consuming process of learning how to dissect out perineal tissues from rat fetuses at different stages of development and to do it in a reproducible manner. In contrast to organs such as the testis, the border of these tissues are not well defined, making it difficult to consistently isolate the same amount of tissue during dissections, especially when moving between different developmental stages. Furthermore, the tissue samples are heterogeneous as they contain both muscles, connective

tissues and skin. As a result, transcriptomics analysis of the entire tissue sample will provide an averaged picture of the gene expression in many different cells types with different differentiation status. This makes it difficult to discern the complex mechanisms that are ultimately responsible for the sexually dimorphic development of the perineum. This could explain why effects on gene expression of *Esr1, Padi2, Wnt2* and *Sfrp4* could not consistently be reproduced in the studies presented in chapter 4 and 5. It is possible that it is only a subset of cells within the perineum that express the factors of the Wnt and estrogen signaling pathway. It is also possible that other contributing pathways could be identified if the sample could be subdivided based cell type. One way to resolve this is by single-cell sequencing that provides gene expression information at single cell resolution and therefore present a way to identify cell types within the samples by clustering methodologies (Shapiro et al, 2013). This method presents an exciting possibility to map the developmental trajectories in the perineum and how these processes are affected in males with short AGD. A great example on the use of single-cell sequencing to track cell differentiation has been provided by Stévant et al. who used single cell sequencing to delineate sex fate determination of gonadal somatic cells (Stevant et al, 2019).

Ex vivo cultures of the perineum were conducted (chapter 5) in the hope that a more controlled experimental setup could reduce sample variation and make it easier to detect transcriptional changes following chemical exposure. If successful, this method could also provide a medium throughput method for screening chemically-induced effects on the perineum. A method for growing the genital tubercle in *ex vivo* culture has been established by the group of Professor Andrew Pask at Melbourne University, Australia (Govers et al, 2019), whom also taught us the experimental set-up. In their experience, it is essential to maintain the perineal tissues attached to the genital tubercle in culture in order for the genital tubercle to survive and grow. Based on their skill and experience from mice we implemented a similar protocol in our own lab for rats. There were, however, important differences in the setup including model species (mouse vs rat), duration of tissue culture, culture medium and the O_2 concentration at which the cultures were kept. It is clear that so many alternating factors makes it very difficult to discern which factors are determining in the success of the culture. This highlights a major limitation to our study, namely that we do not have a method for determining whether or not the culture is successful in recapitulating the *in vivo* situation. Due to the variety of cell types present in the perineal tissues, *ex vivo* culture may induce growth of e.g. cartilage or mesenchyme rather than muscle and end up comprising cell types that are not representative of the tissue *in vivo*. As we do not have a clear reference to how the tissue composition should be based on the *in vivo* situation, it is

difficult to identify the shortcomings of our *ex vivo* model. Also here, single-cell sequencing could provide a great tool to determine the relative contribution of different cell populations *in vivo* and relate this to those present following *ex vivo* culture.

7.8 Perspectives

As sequencing methods have become cheaper and faster during the course of this PhD, alternatives to microarray analysis are now more accessible. An advantage to the low cost of bulk-RNA barcoding (BRB) and sequencing (Alpern et al, 2019) is that it enables us to sequence more samples from each experiment. This will in future studies increase the statistical power and the possibility to identify the transcripts that are consistently altered. At present, we are performing BRB sequencing on the perineal tissues and the genital tubercle isolated at GD17 and GD21 perineum from Sprague Dawley rat fetuses following *in utero* exposure to the agricultural fungicide, triticonazole. Triticonazole has recently been shown to have AR antagonistic activity *in vitro* and to reduce AGD *in vivo* (Draskau et al, 2019). As this is the same mechanism of action as enzalutamide, vinclozolin and procymidone used in the present study, the hope is that we can reproduce our previous findings, focusing especially on the Wnt and estrogen related factors.

A question that remains to be answered is whether there are other signaling pathways that, instead of or in parallel to androgen signaling, can explain the effects seen on AGD following exposure to chemicals with a non-androgenic mode of action. The identification of sexually dimorphic expression of Wnt and estrogen related factors during perineal growth in rats, provides excellent targets for further examination with follow-up studies. Examples of compounds for such future studies could be bisphenol A and butyl paraben that are assumed to have an estrogenic mode of action and in some cases induce short male AGD (Boberg et al, 2016; Christiansen et al, 2014; Zhang et al, 2014), mild analgesics that also induce short male AGD (Holm et al, 2015; Kristensen et al, 2011) but have a less defined mode of action or tebuconazole and propiconazole that in some studies induce a longer male AGD (Goetz et al, 2007; Taxvig et al, 2007). *In utero* exposure to these chemicals could be used to determine whether Wnt and estrogen signaling are implicated in their effects on AGD or if the effects are driven by entirely different pathways. Such future analysis should be based on single cell sequencing to address the issue of tissue heterogeneity.

7.9 Conclusion

There is a need for detailed mechanistic knowledge on how chemicals with different modes of action act on the perineum to affect AGD outcome. This is necessary if we are to construct comprehensive AOPs including all relevant molecular initiating events. We identified Wnt and estrogen signaling as pathways that are sexually dimorphic in the perineum and affected by anti-androgenic exposure during fetal life. Together with previous literature, the preliminary results suggest that sex-specific differences in proliferation and differentiation of cells in the perineum determine the final size of the perineal muscles and contribute to the sexually dimorphic development of AGD. It is possible that Wnt and estrogen signaling may modulate AGD outcome by affecting these processes. Future investigations should address to what extent these pathways contribute to the AGD outcome and whether they are implicated in the effects on AGD induced by chemicals with a non-anti-androgenic mode of action.

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Supplementary material to manuscript I

8.1 Supplementary material

Manuscript I – Supplementary material

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Contents:

Supplemental Table S1

This table is available at a higher resolution at https://doi.org/10.1007/s00204-018-2350-5

Supplementary Table S1: Summary of rat toxicity studies reporting on AGD measurements following gestational exposure to phthalates and other compunds. AGD data after in utero exposure to various substances and the dose at which maximum shorter mean AGD was observed. In many instances, percentage shorter AGD was estimated from published graphs, as raw data was not available.* = Approximation from graph; - = Not assessed;
Abbreviations: **DDE** (DDT metabolite, dichlorodiphenyl- dichloroethylene),**TCDD** (2,3,7,8-tetrachlorodibenzo-p-dioxin),**HBM** (2-Hydroxy-4-Methoxybenzone), **OMC** (Octyl Methoxycinnamate), **DMP** (dimethyl phthalate), **DEP** (diethyl phthalate), **DBP** (dibutyl phthalate), **MBuP** (monobutyl phthalate), **DiBP** (di-isobutyl phthalate), **DEHP** (diethylhexyl phthalate), **DHP** (di-n-hexyl phthalate), **DCHP** (dicyclohexyl phthalate), **BBP** (benzyl butyl phthalate), **MBeP** (monobenzyl phthalate), **DnHP** (di-n-hexyl phthalate), **DHPP** (di-n-heptyl phthalate), **DiHP** (di-isoheptyl phthalate), **DnOP** (di-n-octyl phthalate), **DOTP** (dioctyl terephthalate), **DiNP** (di-isononyl phthalate), **DUDP** (diundecyl phthalate), **DTDP** (ditridecyl phthalate).

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
Aspirin (acetylsalicylic acid)	0; 150; 400	GD11-14	Subcutaneous injection	38	-	400	150	No effect	Mice / B10.A	GD18	N		Gupta&Goldman 1986
acetylsalicylic acid	0; 150; 200; 250	GD13-22	Oral gavage	No effect	No effect	-	-	-	Rat / Wistar	GD22	N (but AGDi calculated)	Decreased fetal weight	Kristensen et al 2011
Aniline	0; 31 ;93	GD7-delivery	Oral gavage	-	20*	93	93	-	Mouse / C57BL/6JBomTac	Week 4	N (but AGDi calculated)		Holm et al. 2015
BBP	0; 750; 3750; 11250ppm. Calculated intake: 50; 250 and 750 mg/kg/day	10 weeks before mating through mating, gestation, lactation and until weaning. F1 exposed through feed after weaning	Through feed	9	-	750	250	No effect	Rat / Sprague-Dawley	PND 0	Y	Reduced birth weight, nipple retention, delayed preputial separation, lower mating and fertility index, reduced absolute weight of epididymis and seminal vesicle, reduced absolute and relative weight of testes and prostate, reduced sperm concentration and motility, increased incidence of reproductive tract malformations	Tyl et al 2004
BBP	0; 500	GD 14-18	oral gavage	13	-	500	500	-	Rat / Sprague-Dawley	PND 2	Y	Nipple retention	Hotchkiss et al. 2004
BBP	0; 250; 500; 1000	GD15-17	Oral gavage	38*	29*	1000	500	No effect	Rat/Wistar	GD21	N (but AGDi calculated)	Undescended testes, decreased fetal weight	Ema & Miyawaki 2002
BBP	0; 750	GD14-PND3	Oral gavage	30	-	750	750	No effect	Rat / Sprague Dawley	PND 2	Y	Reduced birth weight, nipple retention, reduced weight of reproductive organs, genital malformations	Gray et al. 2000
BBP	0;20;100;500	2 weeks premating, through mating, gestation, lactation until PP21	Oral gavage	8	-	500	500	Increased	Rat / Sprague Dawley	PND 0	N	Reduced birth weight, reduced weight of testis and epididymis, decreased number of spermatocytes, delayed preputial separation.	Nagao et al. 2000

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
Benzophenone	0; 100; 450; 2000ppm. Calculated intake: 0; 6.445; 29.01; 130.0 mg/kg for males and 0; 8.379; 38.15 and 166.5mg/kg for females	F0 were exposed from 5wk of age for 10 weeks. Exposure during gestation not stated clearly	Through feed	No effect	No effect	-	-	Decreased AGD and AGDi at 100 and 450ppm	Rat / Sprague dawley	PND4	No (but AGDi calculated)	Reduced birthweight	Hoshino et al 2005b
BPA	0; 0.0025; 0.025	GD6-21	Oral gavage	No effect	No effect	-	-	No effect	Rat / Sprague dawley	PND1	No (but AGDi calculated)		Ferguson et al 2011
BPA	0; 0.025; 0.250; 5; 50	GD7-PND22 (excluding day of delivery)	Oral gavage	7	-	0.25	0.25	Decreased	Rat / Wistar	PND 1	Y	Nipple retention	Christiansen et al. 2014
BPA	0; 60; 600; 3000ppm. Calculated intake: gestation: 0; 5.1; 49.1; 231.8 mg/kg/day Lactation: 0; 8.5; 80.2; 384.4mg/kg/day	GD15-PND10	Through feed	No effect	-	-	-	No effect	Rat / Sprague dawley	PND2	N	Decreased pup weight	Takagi et al 2004
BPA	0; 0.02; 0.1; 50	GD6-21	Oral gavage	No effect	No effect	-	-	No effect	Rat / AP (Wistar derived)	PND1	N (but no effect on body weight)		Tinwell et al 2002
BPA	0; 0.02; 0.1; 50	GD6-21	Oral gavage	No effect	No effect	-	-	No effect	Rat / Sprague dawley	PND1	N (but no effect on body weight)		Tinwell et al 2002
BPA	0; 0.002; 0,02; 0,2	GD7-PND18	Oral gavage	No effect	-	-	-	-	Rat / Long Evans	PND 2	N		Howdeshell et al. 2008
Butyl paraben	0; 10; 100; 500	GD7-21 + PND1-22	Oral gavage + Lactation	7	6	500	100	Decreased	Rat / Wistar	PND 1	Y	Reduced sperm count, reduced prostate weight	Boberg et al. 2016
Butyl paraben	0; 64; 160; 400; 1000	GD7-PND21	Oral gavage	16	-	1000	400	-	Rat / Wistar	PND 1	N	Reduced bith and pup weight. Delayed preputial separation, reduced weight of testis, epididymis and seminal vesicles, reduced sperm production.	Zhang et al 2014
Butyl paraben	0; 600	GD 7-21	oral gavage	No effect	No effect	600 (only dose tested)	-	No effect	Rat / Wistar	GD 21	Y		Boberg et al. 2008
DBP	7600ppm. Calculated intake: Gestation 642. Lactation: 1138	GD12-PND14	Through feed	11 (PND2), 9 (PND14), No effect (PND49)	10 (PND2), 8(PND14), No effect (PND49)	7600	7600	-	Rat / Sprague Dawley	PND2	?	Reduced testis weight, nipple retention, histological changes in testis, reduced absolute and relative weight of seminal vesicles and LABC, Reduced relative weight of ventral prostate, malformations of the male reproductive tract.	Clewell et al 2013

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
DBP	0; 500	GD14-15 or GD15-16 or GD16-17 or GD17-18 or GD18-19 or GD19-20	Oral gavage	20*	-	500	500 (lowest tested)	-	Rat / Sprague Dawley	PND 1	Y	Increased testis and epididymis weights, Nipple retention, malformations of the reproductive tract.	Carruthers & Foster 2005
DBP	0; 500	e13.5-20.5 or e11.5-20.5 or e13.5-15.5 or e15.5-17.5 or e19.5-20.5	Oral gavage	20*	-	500	500 (lowest tested)	-	Rat / Wistar	GD21.5	N		Scott el al. 2008
DBP	0; 500	GD14-18	Oral gavage	9	-	500	500 (lowest tested)	-	Rat / Sprague-Dawley	PND 3	N	Nipple retention	Howdeshell et al. 2007
DBP	0; 100; 500	GD 12- 21	Oral gavage	14	-	500	500	-	Rat / Sprague-Dawley	PND 1	Y	Nipple retention, histological changes in testis	Barlow et al. 2004
DBP	0; 351; 555; 661 (calculated from 0,5%; 1%; 2% in feed)	GD11-20	Through feed	43*	26* (AGD/BW	661	555	No effect reported	Rat/Wistar	GD21	N (but also AGD/BW)	Undescended testes	Ema et al. 1998
DBP	0; 500; 1000; 1500	GD 12-14; GD 18-20; (	Oral gavage	48*	26* (AGD/BW	1500	500	No effect	Rat/Wistar	GD21	N (but also AGD/BW)	Undescended testes, decreased fetal weight	Ema et al. 2000
DBP	0; 250; 500; 750; 1000	GD 14-18	Oral gavage	-	9%	750	500 (6% decrease)	-	Rat / Sprague Dawley	PND 1	Y	Decreased pup bodyweight, increased incidence of cryptorchidism and hypospadias, decreased weight of reproductive organs	Jiang et al. 2007
DBP	0; 850	GD 12-18	Oral gavage	20	-	850	-	-	Rat / Sprague Dawl	PND 1	N	Swollen abdominal features with absence of scrotum and testis in the perineum	Jiang et al. 2011
DBP	0; 850	GD 12-18	Oral gavage	-	6	850	-	-	Rat / Sprague Dawl	PND 1	Y		Jiang et al. 2015b
DBP	Approximately (doses originally given in ppm) 0; 1.5; 14.4; 148.2; 712.3	GD15-PND21	Through feed	19	-	712.3 NB! At all other doses AGD increases	712.3	No effect	Rat/CD®(SD)IGS	PND2	N	Increased pup weight. Nipple retention, testicular dysgenesis, epididymal lesions, increased relative and absolute weight of testis and ventral prostate, reduced number of spermatocytes	Lee et al. 2004
DBP	0; 100; 300; 900	E12.5-20.5	Oral gavage	27	-	900	300	-	Rat / Wistar	PND 2	N	Genital malformations, reduced testis weight, testicular dysgenesis	Li et al . 2015
DBP	0; 850	GD12-18	Oral gavage	20*	-	850	850 (only dose tested)	-	Rat / Sprague Dawley	PND 1	N	Reduced birth weight, anorectal malformations	Liu et al. 2016

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
DBP	0; 100; 500	GD13-21	Oral route	12	12	500	100	No effect	Rat / Wistar	GD21	N (but AGDI calculated)	Nipple retention, testicular dysgenesis	Martino-Andrade et al. 2009
DBP	0; 250; 500; 750	GD3-PND20 (except parturition and the following day)	Oral gavage	24	-	750	500	No effect	Rat / Sprague Dawley	PND 1	N	Hypospadias, absent or underdeveloped prostate, epididymis, seminal vesicles and testicles	Mylchreest et al. 1998
DBP	0; 100; 250; 500	GD12-21	Oral gavage	24	-	500	250	-	Rat / Sprague Dawley	PND 1	N	Nipple retention, delayed preputial separation, reduced weight of seminal vesicle, epididymis and testis, testicular dysgenesis	Mylchreest et al. 1999
DBP	0; 500	GD12-21	Oral gavage	24	-	500	500 (only dose tested)	No effect	Rat / Sprague Dawley	PND 1	Y	Reduced birth weight, nipple retention, testicular dysgenesis, genital malformations, reduced weight of testis, epididymis, seminal vesicles and prostate, delayed preputial separation.	Saillenfait et al. 2008
DBP	0; 100; 500	GD15-PND21	Oral gavage	28	21	500	100	-	Rat / Wistar	PND1	N (but AGD/BW calculated)		de Mello Santos et al. 2017
DBP	0; 500	e13.5-20.5	Oral gavage	No effect	-	-	-	-	Rat / Wistar	GD21	N	Testicular dysgenesis	Scott et al. 2007
DBP	0; 500; 750	e13.5-20.5 or e15-5-1	Oral gavage	36*	-	750	500 (lowest tested)	-	Rat / Wistar	GD 21	N	Testicular dysgenesis	Van den Driesche et al. 2012
DBP	0; 750	e13.5–21.5 or e15.5–:	Oral gavage	20*	-	750	750	-	Rat / Wistar	GD 21	N	Testicular dysgenesis, genital malformations	Van den Driesche et al. 2017
DBP	0; 500	GD16-19	Oral gavage	12	-	500	500	-	Rat / Long Evans	PND 2	Y	Nipple retention, reduced weight of seminal vesicle, ventral prostate, LABC muscles.	Wolf et al. 1999
DBP	0; 500	GD14-PND3		25	-	500	500	-	Rat / Sprague Dawley	PND 2	Y	Nipple retention. Epididymal and testicular malformations. Reduced weight of ventral prostate, epididymis, testis, glans penis and the BC/LA muscles.	Wolf et al. 1999
DCHP	0; 20; 100; 500	GD6-19	Oral gavage	27*	26*	500	20	-	Rat / Wistar	GD20	N (but AGDi calculated)	Increased pup bodywieght. Testicular dysgenesis	Aydogan Ahbab and Barlas 2015
DCHP	0;250;500;750	GD6-20	Oral gavage	17	13	750	250	No effect	Rat / Sprague Dawley	GD21	Y	Reduced birth weight	Saillenfait et al. 2009a

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
DCHP	0; 240; 1200; 6000ppm. Calculated intake: Premating: 0; 17.7; 88.83; 434.6. Gestation: 0; 14.3; 69.77; 349.0. Lactation: 37.92; 191.6; 932.8	F0 were exposed from 5wk of age until necropsy	Through feed	6	7	6000ppm	6000ppm	No effect	Rat / Sprague Dawley	PND4	N (but AGDI calculated)	Reduced birth weight, reduced prostate weight, atrophy of seminiferous tubules, reduced sperm count	Hoshino et al. 2005
DDE	0; 1; 10; 50; 100; 200	GD14-18	Oral gavage	-	11 (AGD/Crown rump length)	50-200	50	-	Rat / Holtzman	PND 1	N (but AGD/crown rump length calculated)	Decreased fetal weight. Nipple retention, delayed preputial separation, reduced weight of prostate, decreased sperm count	Loeffler and Peterson 1999
DDE	0; 100	GD14-18	Oral gavage (not clearly stated)	9	-	100	100	-	Rat / Sprague Dawley	PND 2 (not clearly stated)	N	Nipple retention, reduced weight of glans penis, epididymis, ventral prostate and LABC muscles.	Wolf et al. 1999
DDE	0; 100	GD14-18	Oral gavage (not clearly stated)	6	-	100	100	-	Rat / Long Evans	PND 2 (not clearly stated)	N	Nipple retention, reduced weight of ventral prostate	Wolf et al. 1999
DEHP	0; 30; 300	GD8-PND21 (with exc	Oral gavage	-	5*	300	300	No effect	Rat / Sprague Dawley	PND 3	N (but AGDI calculated)	Histological changes in testis	Nardelli et al 2017
DEHP	0; 3; 15; 30	GD7-PND 16 (with exception of PND0)	Oral gavage	-	No effect	-	-	No effect	Rat / Wistar	PND 0	Y		Christiansen et al. 2009
DEHP	0; 750	GD13-21	Oral gavage (also treated with vehicle by subcutaneous injection)	18	17	750	750	-	Rat / Wistar	PND 4	N (but AGDI calculated)	Reduced weight of male reproductive organs	Kita et al. 2016
DEHP	0;500	GD14-18	Oral gavage	10	-	500	500 (lowest tested)	-	Rat / Sprague-Dawley	PND 3	N	Nipple retention	Howdeshell et al. 2007
DEHP	0; 3; 10; 30; 100; 300; 600; 900	GD7-PND 16 (with exception of PND0)	Oral gavage	14	-	900	10	-	Rat / Wistar	PND 1	Y	Nipple retention, reduced weight of LABC muscles and prostate, mild external genitalia dysgenesis, histological changes in testis	Christiansen et al. 2010
DEHP	0; 300; 750	GD 7-PND 17	Oral Gavage	17	-	750	300	-	Rat / Wistar	PND 3	Y	Nipple retention, decreased weight of ventral prostate and LABC muscles, alterations in testis morphology	Jarfelt et al . 2005

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
DEHP	0; 750	GD14-PND3	Oral gavage	30	-	750	750	No effect	Rat / Sprague Dawley	PND 2	Y	Reduced birth weight, nipple retention, reduced weight of reproductive organs, genital malformations	Gray et al. 2000
DEHP	0; 500; 750; 1000	GD12-19	Oral gavage	30	11 (AGD/BW)	1000	500	-	Rat / Sprague Dawley	PND 1	N (but AGD/BW calculated)	Reduced birth weight, reduced adult penile length, hypospadias.	Li et al. 2013
DEHP	0; 10; 750	GD12.5-PND21	Oral gavage	18	-	750	750	-	Rat / Long Evans	PND 2	N	Reduced pup weight, testicular dysgenesis	Lin et al. 2009
DEHP	0; 150	GD13-21	Oral route	No effect	No effect	(150 tested)	-	No effect	Rat / Wistar	GD21	N	Reduced seminal vesicle weight	Martino-Andrade et al. 2009
DEHP	0; 500	GD12-21	Oral gavage	18	18	500	500	-	Rat / Sprague Dawley	PND 1	Y	Nipple retention. Genital malformations	Saillenfait et al. 2009b
DEHP	0; 750	GD14-PND3	Oral gavage	34	-	750	750	-	Rat / Sprague Dawley	PND 2	Y	Reduced birth weight, nipple retention, reduced weight of seminal vesicle, ventral prostate, epididymis, testis, glans penis and LABC muscles	Wolf et al. 1999
DEHP	0; 375; 750; 1500; 3000 (due to immense toxicity no pup data was measured in the highest dose group)	GD3-PND21	Oral gavage	27*	-	1500	750	No effect	Rat / Sprague-Dawley	PND 1	Y (AGDI also calculated but data is not shown)	Nipple retention, delayed preputial separation	Moore et al. 2001
DEP	0; 750	GD14-PND3	Oral gavage	No effect	-	No effect	No effect	No effect	Rat / Sprague Dawley	PND 2	Y		Gray et al. 2000
Dexamethasone	0; 0.1	e13.5-20.5	Subcutaneous injection	10*	-	0.1	0.1 (lowest tested)	-	Rat / Wistar	GD 21	N		Van den Driesche et al. 2012
DHP	0; 20; 100; 500	GD6-19	Oral gavage	20*	23*	500	20	-	Rat / Wistar	GD 20	N (but AGDI calculated)	Reduced pup weight. Testicular dysgenesis	Aydogan Ahbab and Barlas 2015
DHPP	0; 250; 500; 1000	GD6-20	Oral gavage	11	10	1000	1000	No effect	Rat / Sprague Dawley	GD21	Y		Saillenfait et al. 2011
DiBP	0; 600	GD 7-20/21 or GD 7- 1	Oral gavage	14*	9*	600	600	Increased	Rat / Wistar	GD 21	y		Borch et al. 2006
DiBP	0; 125; 250; 500; 625	GD12-21	Oral gavage	22	-	625	250	No effect	Rat / Sprague Dawley	PND 1	Y	Reduced birth weight, nipple retention, testicular dysgenesis, genital malformations, reduced weight of testis, epididymis, seminal vesicles and prostate, delayed preputial separation	Saillenfait et al. 2008
DiBP	0; 250	GD13-19	Oral gavage	No effect	5	250	250	-	Rat / Sprague Dawley	GD 19	N (but AGD/BW calculated)		Saillenfait et al. 2017

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
DIHP	0; 1000; 4500; 8000PPM Calculated mean intake: Prior to breeding: 0; 89; 406; 726. Gestation: 0; 64; 304; 532. Lactation: 0; 162; 716; 1289	70 days to mating, thr	Through feed	15	-	8000ppm ca. 532mg/kg/day	8000ppm ca 532mg/kg/day	No effect	Rat / Sprague Dawley	PND 1	AGDi also significantly different buit data not shown	Nipple retention, genital malformations, delayed preputial separation, reduced sperm count	McKee et al 2006
DINP	0; 760; 3800; 11400ppm. Calculated intake: GD13-20: 0; 56; 288; 720. PND2-14: 0; 109; 555; 1513; 1138	GD12-PND14	Through feed	No effect (PND2), 16 (PND14), No effect (PND49)	No effect (PND2), 7 (PND14), No effect (PND49)	11400	11400	-	Rat / Sprague Dawley	PND 2, PND 14, PND 49		Reduced pup weight, histological changes in testis, reduced absolute LABC muscle weight	Clewell et al 2013
DINP	0; 400; 4000; 20000ppm. Calculated intake: Gestation: 0; 30.7M; 306.7; 1164.5. Lactation: 0; 66.2; 656.7; 2656.7	GD15-PND 10	Through feed	No effect	-	-	-	No effect	Rat / Sprague Dawley	PND 2	N	Reduced absolute and relative testes weight, histological changes in testis	Masutomi et al 2003
DINP	0; 300; 600; 750; 900	GD 7 -PND 17	Oral gavage	8	6	900	900	No effect	Rat / Wistar	PND 1	Y	Nipple retention, reduced sperm motility, increased sperm count	Boberg et al. 2011
DiNP	0; 750	GD14-PND3	Oral gavage	No effect	No effect	-	-	No effect	Rat / Sprague Dawley	PND 2	Y	Nipple retention, genital malformations	Gray et al. 2000
DMP	0; 750	GD14-PND3	Oral gavage	No effect	No effect	-	-	No effect	Rat / Sprague Dawley	PND 2	Y		Gray et al. 2000
DnHP	0; 250; 500; 750	GD6-20	Oral gavage	35	31	750	250	Decreased	Rat / Sprague Dawley	GD21	Y	Reduced birth weight, genital malformations	Saillenfait et al. 2009a
DnHP	0;50;125;250;500	GD12-21	Oral gavage	18	18	500	125	-	Rat / Sprague Dawley	PND 1	Y	Nipple retention, genital malformations, testicular dysgenesis	Saillenfait et al. 2009b
DnOP	0; 250; 500; 1000	GD6-20	Oral gavage	No effect	No effect	-	-	-	Rat / Sprague Dawley	GD21	Y	Increased fetal weight	Saillenfait et al. 2011
DOTP	0; 750	GD14-PND3	Oral gavage	No effect	No effect	-	-	No effect	Rat / Sprague Dawley	PND 2	Y		Gray et al. 2000
DTDP	0; 250; 500; 1000	GD6-20	Oral gavage	No effect	No effect	-		No effect	Rat / Sprague Dawley	GD21	Y		Saillenfait et al. 2013

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
DUDP	0; 250; 500; 1000	GD6-20	Oral gavage	No effect	4	500	500	No effect	Rat / Sprague Dawley	GD21	Y		Saillenfait et al. 2013
Epoxiconazole	0; 50	GD7-21	Oral gavage	No effect	No effect	-	-	No effect	Rat / Wistar	GD21	Y	Increased fetal weight	Taxvig et al 2008
Epoxiconazole	0; 3.75; 15	GD7-21 + day after birth - PD16	Oral gavage	5 increased (only effect at low dose)	5 increased	3.75	3.75	Increased (only effect at low dose)	Rat / Not stated	PD1	Y		Hass et al 2012
Epoxiconazole	0; 15; 50	GD7-PND16	Oral gavage	GD21: No effect. PND0: 7 Increased (only at low dose)	GD21: 10 increased (only at low dose). PND 0: No effect	15 (both time points)	15 (both time points)	Increased (PND 0: only effect at low dose)	Rat / Wistar	GD21 + PND0	Y	Decreased fetal weight. Increased birth weight.	Taxvig et al 2007
Ethinyl estradiol	0; 0.005; 0.01	oral gavage	GD6-21	No effect	No effect	-	-	No effect	Rat / Sprague dawley	PND1	AGDi calculated	Decreased birth weight. No other morphological reproductive endpoints measured	Ferguson et al 2011
Ethinyl estradiol	0; 0.005; 0.015; 0.05	GD 7-PND 22	Oral gavage	No effect	No effect	No effect	-	Increased	Rat / Wistar	PND 1	Y		Mandrup et al 2013
Ethinyl estradiol	0; 0.00005; 0.0005; 0.0015; 0.005; 0.015; 0.050	GD7-PND18	Oral gavage	No effect	-	-	-	-	Rat / Long Evans	PND2	N	Reduced weight of androgen dependent tissues, reduced sperm count	Howdeshell et al. 2008
Fenitrothion	0; 5; 10; 15; 20; 25	oral gavage	GD12-21	16	-	25	25 (at 20mg/kg/day AGD reduced 8%= p=0.05)	No effect	Rat/Sprague dawley	PND1	Y	Nipple retention	Turner et al 2002
Finasteride	0; 0.01; 0.1	GD 7-PND16	Oral gavage	-	9	0.1	0.1	-	Rat / Wistar	PND 0	Y	Nipple retention, decreased epididymal weight	Christiansen et al. 2009
Finasteride	0; 0.01; 0.1; 10; 100	GD12-21	Oral gavage	33*	-	100	0.1	-	Rat / CRL:CD(SD)BR	PND 1	Y	Nipple retention, reduced prostate weights, genital malformations	Bowman et al. 2003
Flutamide	0; 20	GD13-21	Oral gavage (also treated with vehicle by subcutaneous injection)	44	41	20	20	-	Rat / Wistar	PND 4	N (but AGDi calculated)	Reduced weight of male reproductive organs	Kita et al. 2016
Flutamide	0; 50	GD16, -17, -18 or 19	Oral gavage	16	-	50	50	-	Rat / Sprague-Dawley	PND1	Y	Nipple retention, malformations of the reproductive tract	Foster & Harris 2005
Flutamide	0; 100	e15.5-21.5 or e15.5-17.5 or e19.5-21-5	Oral gavage	50*	-	100	100	-	Rat / Wistar	GD21.5	N		Welsh et al. 2007
Flutamide	0; 6.25; 12.5; 25; 50	GD12-21	Oral gavage	53	-	50	6.25	-	Rat / CRL:CD(SD)BR	PND 1	Y	Nipple retention, malformations of the reproductive tract, reduced weight of prostate, testis, LABC, seminal vesicle, and epididymis, Absent bulbourethral glands	McIntyre et al. 2001
Flutamide	0; 0.5; 1.0; 2.0; 4.0; 8.0; 16	GD 7-PND 16	Oral Gavage	-	42*	16	<0.5 (NOAEL)	-	Rat / Wistar	PND 1	Y	Nipple retention	Hass et al. 2007

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
Flutamide	0;100	GD12-21	Oral gavage	55*	-	100	100 (lowest tested)	-	Rat / Sprague Dawley	PND 1	N	Nipple retention, genital malformations, absent prostate, reduced testis weight, vaginal pouch, testicular dysgenesis	Mylchreest et al. 1999
Flutamide	0; 100	e15.5-20.5	Oral gavage	33*	-	100	100	-	Rat / Wistar	GD21	N		Scott et al. 2007
Genistein	0; 20; 200; 1000ppm. Calculated intake: Gestation: 0; 1.3; 13.7; 66.6. Lactation: 0; 2.1; 23.0; 113.1	GD15-PND 10	Through feed	No effect	-	-	-	No effect	Rat / Sprague Dawley	PND2	N		Masutomi et al 2003
HBM	1000, 3000, 10,000, 25,000, or 50,000 ppm. Calculated intake: Perinatal GD3-10: 0; 74.9; 223.2; 712.5; 2041.8; 3749.1. GD10-13: 0; 65.7; 203.1; 626.8; 1747.5; 3214.7. GD13-20: 0; 63.2; 195.0; 673.2; 1605.5; 3380.6. PND0-7: 0; 66.6; 249.5; 756.2; 1864.7; 3756.8. PND7-14: 0; 88.4; 393.0; 1173.8; 2793.3; 5274.2. PND14-23 0; 195; 656.1; 2257.7; 4650.7; 7178.5	Through feed	GD6-PND23	-	No effect	-	-	No effect	Rat / Sprague Dawley	PND1	AGDi calculated	Reduced fetal weight. Histological changes in testis	Nakamura et al 2015
Ketoconazole	0; 50	GD7-21	Oral gavage	8	11	50	50	Decreased	Rat / Wistar	GD21	Y		Taxvig et al 2008
Ketoconazole	0; 12.5; 25; 50	GD14-PND3	Oral gavage	No effect	-	-	-	-	Rat / Long Evans	PND 2	N	Reduced weight of testis, seminal vesicle and epididymis.	Wolf et al. 1999
Lindane	0; 10; 60; 300ppm. Calculated intake: Gestation: 0; 0.5730; 3.389; 16.55. Lactation: 09; 1.525; 8.941;	11 weeks before mating through mating, gestation, lactation and until weaning. F1 treated after weaning	Through feed	No effect	No effect	-	-	Decreased but only in 1 (10ppm) of 4 doses. No dose dependency	Rat / Sprague dawley	PND 4	N (but AGDI calculated)	Decreased birth weight	Matsuura et al 2005b
Linuron	0; 75	GD 14-18	Oral gavage	25	-	75	75	-	Rat / Sprague-Dawley	PND 2	Y	Nipple retention	Hotchkiss et al. 2004

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
Linuron	0; 12.5; 25;50	GD 12-21	Oral gavage	No effect	-	50	-	-	Rat / Sprague-Dawley	PND 1	Y	Nipple retention, testicular and epididymal lesions	McIntyre et al. 2000
Linuron	0; 50	GD 12-21	Oral gavage	8	-	50	50	No effect	Rat / Sprague-Dawley	PND 1	Y	Nipple retention, enlarged testes, testicular and epididymal abnormalities	McIntyre et al. 2002
Linuron	0; 100	GD14-GD18	Oral gavage	31	-	100	100	-	Rat / Sprague Dawley	PND 2	Y	Reduced birth weight, nipple retention, epididymal and testicular malformations, reduced weight of ventral prostate, epididymis, testis, glans penis and LABC muscles.	Wolf et al. 1999
Mancozeb	0; 6.25; 25	GD7-21 + day after birth - PD16	Oral gavage	No effect	No effect	-	-	No effect	Rat / Not stated	PD1	Y	Nipple retention	Hass et al 2012
MBeP	0; 167; 250; 375	GD15-17	Oral gavage	30*	29*	375	250	No effect	Rat / Wistar	GD21	N (but AGDi calculated)	Decreased fetal weight, undescended testes	Ema et al. 2003
MBuP	0; 250; 500; 750	GD15-17	Oral gavage	39*	29*	750	250	No effect	Rat / Wistar	GD21	N (but AGDi calculated)	Decreased fetal weight, undescended testes	Ema & Miyawaki 2001
Methoxychlor	0; 24; 240; 1200ppm Gestation: 0; 1.9; 18.7; 81.4. Lactation: 0; 3.8; 35.9; 167.6	GD15-PND 10	Through feed	No effect	-	-	-	No effect	Rat / Sprague Dawley	PND2	N	Delayed preputial separation, reduced absolute and relative testes weight, reduced prostate fluid	Masutomi et al 2003
Myclobutanil	0; 100; 500; 2000ppm. Calculated intake 8; 38; 145; mg/kg/day	GD6 throughout gestation and lactation. F1 until necropsy	Through feed	12* increased	-	2000	2000	-	Rat / Wistar	PND0	Y	Increased birth weight at PND0, decreased body weight from PND22 and onward, increased weight of testis and ventral prostate, reduced insemination and fertility indices	Goetz et al 2007

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
Nonylphenol	0; 60; 600; 3000ppm Calculated intake: gestation: 0; 4.8; 48.2; 270.4mg/kg/day. Lactation: 0; 9.1; 87.2; 455.3mg/kg/day	GD15-PND10	Through feed	No effect	-	-	-	Decreased	Rat / Sprague dawley	PND2	N		Takagi et al 2004
OMC	0; 500; 750; 1000	GD7-PND17 (day of delivery excluded)	Oral gavage	No effect	No effect	-	-	No effect	Rat / Wistar	PND1	Y	Decreased birth weight, decreased relative testes and prostate weight, histological alterations in testis and prostate, reduced number of sperm	Axelstad et al 2011
Paracetamol	0; 360	GD7-21 + PND1-22	Oral gavage	-	No effect	-	-	No effect	Rat / Wistar	PND1	Y	Nipple retention, reduced LABC weight	Axelstad et al 2014
Paracetamol (Acetaminophen)	0; 20 (three times a day); 350 (once daily)	e13.5-e17.5	Oral gavage	8*	9*	350	350 (only dose where AGD is measured		Rat / Wistar	e.21.5	AGDi calculated		Van den Drische et al 2015
Paracetamol	0; 50; 150	GD7-delivery	Oral gavage	-	15*	150	150	-	Mouse / C57BL/6JBomTac	Week 10	AGDi calculated		Holm et al. 2015
Paracetamol	0; 150; 250; 350	GD13-21	Oral gavage	9	10.5	150	150	-	Rat / Wistar	GD21	N (but AGDi calculated)		Kristensen et al 2011
Procloraz	0; 8.75; 35	GD7-21 + day after birth - PD16	Oral gavage	No effect	No effect	-	-	Increased	Rat / Not stated	PD1	Y	Nipple retention	Hass et al 2012
Prochloraz	0; 5; 25; 30	GD 7- PND 16	Oral gavage	-	No effect	-	-	-	Rat / Wistar	PND 0	Y	Nipple retention	Christiansen et al. 2009
Prochloraz	0; 30	GD7-PND17	Oral gavage	No effect	No effect	-	-	-	Rat / Wistar	PND 0	N	Nipple retention, decreased bulbourethral weight	Vinggaard et al. 2005
Prochloraz	0; 0.01; 5; 30	from GD6 until PND30	Oral gavage	No effect	No effect	-	-	Increased	Rat / Wistar	PND 1	?	Nipple retention and delayed puberty	Melching-Kollmuss et al. 2017
Prochloraz	0; 50; 150	GD7-16	Oral gavage	-	12	150	50	Increased	Rat / Wistar	PND 0	N	Nipple retention, mild dysgenesis of external genitalia, reduced weight of seminal vesicles and bulbourethral glands	Laier et al. 2006
Prochloraz	0; 31.25; 62.5; 125; 250	GD14-18	Oral gavage	6	-	250	62.5	Increased	Rat / Sprague-Dawley	PND 3	Y but only significant when not analysed with BW as covariate	Reduced bodyweight, nipple retention, malformations of the reproductive tract	Noriega et al. 2005
Procymidone	0; 12.5; 50	GD7-21 + day after birth - PD16	Oral gavage	10	9	50	12.5	No effect	Rat / Not stated	PD1	Y	Nipple retention, genital malformations	Hass et al 2012

Substance	Doses (0; xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
Procymidone	5; 10; 25; 50; 100; 150	GD 7-PND 16	Oral gavage	-	37*	150	25	-	Rat / Wistar	PND 1	Y	Nipple retention	Hass et al. 2007
Procymidone	0; 100	GD14-PND3	Oral gavage?	24	-	100	100	-	Rat / Long Evans	PND 2??	N	Nipple retention, hypospadias, reduced weight of ventral prostate, seminal vesicle and LABC muscles, prostatitis and vesiculitis	Wolf et al. 1999
Propiconazole	0; 50	GD7-21	Oral gavage	No effect	No effect	-	-	No effect	Rat / Wistar	GD21	Y	Increased fetal weight	Taxvig et al 2008
Propiconazole	0; 100; 500; 2500ppm calculated levels were approximately 8; 37; 158 mg/kg/day	GD6 throughout gestation and lactation. F1 until necropsy	Through feed	7* increased	-	2500	2500	-	Rat / Wistar	PND0	Y	Increased testis weight, delayed preputial separation.	Goetz et al 2007
TCDD	0; 0.001	GD15	Oral gavage	6	(No effect at AGD/Crown rump length)	0.1	0.1	-	Rat / Holtzman	PND 1	N	Reduced birth weight, reduced weight of glans penis, ventral prostate and seminal vesicles, delayed preputial separation, reduced sperm production	Bjerke & Peterson 1994
TCDD	0; 0.001	GD8 or -15	Oral gavage	12 (not significant with BW as covariate)	-	0.1	0.1	-	Rat / Long Evans	PND 3 (measured at PND 1, 3, 8 and 22. Not significant at PND 1)	Y	Reduced weight of testes, seminal vesicles and epididymis, decreased sperm count, delayed preputial separation, reduced fertility	Gray et al. 1995
Tebuconazole	0; 50; 100	GD7-PND16	Oral gavage	No effect	GD21: 10 Increased. PND 0: No effect	100	100	Increased	Rat / Wistar	GD21 + PND0	Y	Decreased fetal weight, nipple retention	Taxvig et al 2007
Tebuconazole	0; 50	GD7-21	Oral gavage	No effect	No effect	-	-	No effect	Rat / Wistar	GD21	Y		Taxvig et al 2008
Tebuconazole	0; 12.5; 50	GD7-21 + day after birth - PD16	Oral gavage	No effect	No effect	-	-	Increased	Rat / Not stated	PD1	Y	Nipple retention	Hass et al 2012
Triadimefon	0; 100; 500; 1800ppm Calculated intake 8; 37; 114 mg/kg/day	GD6 throughout gestation and lactation. F1 until necropsy	Through feed	3* increased	-	1800	1800	-	Rat / Wistar	PND0	Y	Reduced pup weight, increased weight of testis and ventral prostate, increased epididymal weight, reduced insemination and fertility indices	Goetz et al 2007

Substance	Doses (0;xx; mg/kg)	Dosing period	Route of administration	AGD max effect (%)	AGDi max effect (%)	Dose at max effect (mg/kg bw/day)	LOAEL (AGD) (mg/kg bw/ day)	Effect on female AGD	Species/Strain	Time of measurement	Bodyweight as covariate	Additional notes	Reference
				Shortened	Shortened						(Y/N)		
Vinclozolin	Target dose: 0; 4; 20; 100. Calculated intake: Gestation: 0; 4.5; 22; 103. Lactation: 0; 8.0; 39; 191	10 weeks old up until weaning. Selected F1 exposed until necropsy.	Through feed	28	22	100	20	No effect at PND1	Rat / Wistar	PND1	N (but AGDi calculated)	Nipple retention	Schneider et al. 2011
Vinclozolin	0; 1.5; 3; 6; 12	GD17-PND3 except PND 0	Oral gavage	No effect	-	-	-	No effect	Rat / Long evans	PND1-PND20 every 4th day	N		Colbert et al 2005
Vinclozolin	0; 40; 200; 1000ppm. Calculated intake mg/kg/day: Gestation: 0; 2.285; 11.22; 58.65. Lactation: 0; 5.946; 29.42; 145.55	10 weeks before mating through mating, gestation, lactation and until weaning. F1 treated after weaning	Through feed	PND 0: 21. PND 4: 28	PND 0:21 PND 4: 26	1000ppm	1000ppm	No effect	Rat / Sprague dawley	PND 0 + PND4	N (but AGDi calculated)	Nipple retention, delayed preputial separation, reduced absolute weight of epididymis, cauda epididymis, seminal vesicles and prostate, reduced relative weight of cauda epididymis, prostate and testes, genital malformations, decrease in prostate fluid, histological changes in testis	Matsuura et al 2005a
Vinclozolin	0; 200	GD14-19	Oral gavage	46	-	200	200 (lowest tested)	No effect	Rat / Sprague-Dawley	PND2	Y	Nipple retention, genital malformations, reduced weight of reproductive organs	Wolf et al. 2004
Vinclozolin	0; 5; 50	GD 7-PND 16	Oral gavage	-	9	50	50	-	Rat / Wistar	PND 0	Y	Nipple retention, reduced weight of epididymis and bulbourethral glands	Christiansen et al. 2009
Vinclozolin	0; 3.125; 6.25; 12.5; 25; 50; 100	GD14-PND3	Oral gavage	28	-	100	3.125	-	Rat / Long-evans	PND2	Y	Nipple retention, malformations of the reproductive tract, reduced ventral prostate weight, reduced fertility	Ostby et al. 1999
Vinclozolin	0; 100; 200	GD14-PND3	Oral gavage	56	-	200	100	Decreased	Rat / Long-evans	PND 1	N	Nipple retention, malformations of the reproductive tract, reduced prostate weight, vaginal pouch	Gray et al. 1994
Vinclozolin	5; 10; 20; 40; 80 160	GD7-16	Oral gavage	-	35	160	5 (NOAEL)	-	Rat / Wistar	PND 1	Y	Nipple retention	Hass et al. 2007

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Supplementary material to manuscript II

Manuscript II – Supplemental material

Schwartz, C. L., Vinggaard, A. M., Christiansen, S., Darde, T. A., Chalmel, F., and Svingen, T. Distinct transcriptional profiles of the female, male and finasteride-induced feminized male anogenital region in rat fetuses. *Tox Sci 2019 169(1), 303–311*

Contents:

Supplementary Figure 1

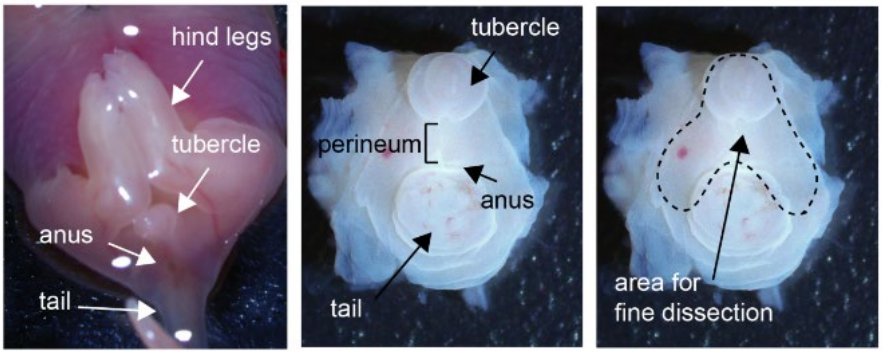

Figure S1: Rat foetuses were collected by caesarean section on GD21 (left panel). For dissection of the desired anogenital tissue, fetuses were placed on their backs and a larger section of the anogenital region removed and hind legs and tail trimmed off (middle panel; magnified). Finer dissection was performed to isolate the muscles and underlying tissue that make up the perineal region plus the genital tubercle (right panel).

Supplementary Figure 2

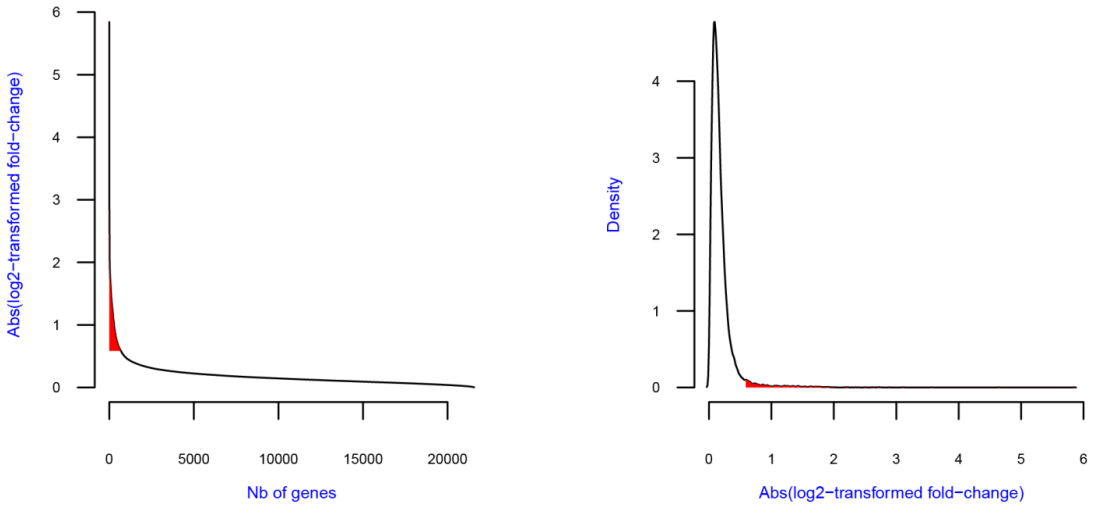

Figure S2: Fold change plots for gene array data. A fold change of at least 1.5 was used for the data analysis.

Supplementary Figure 3

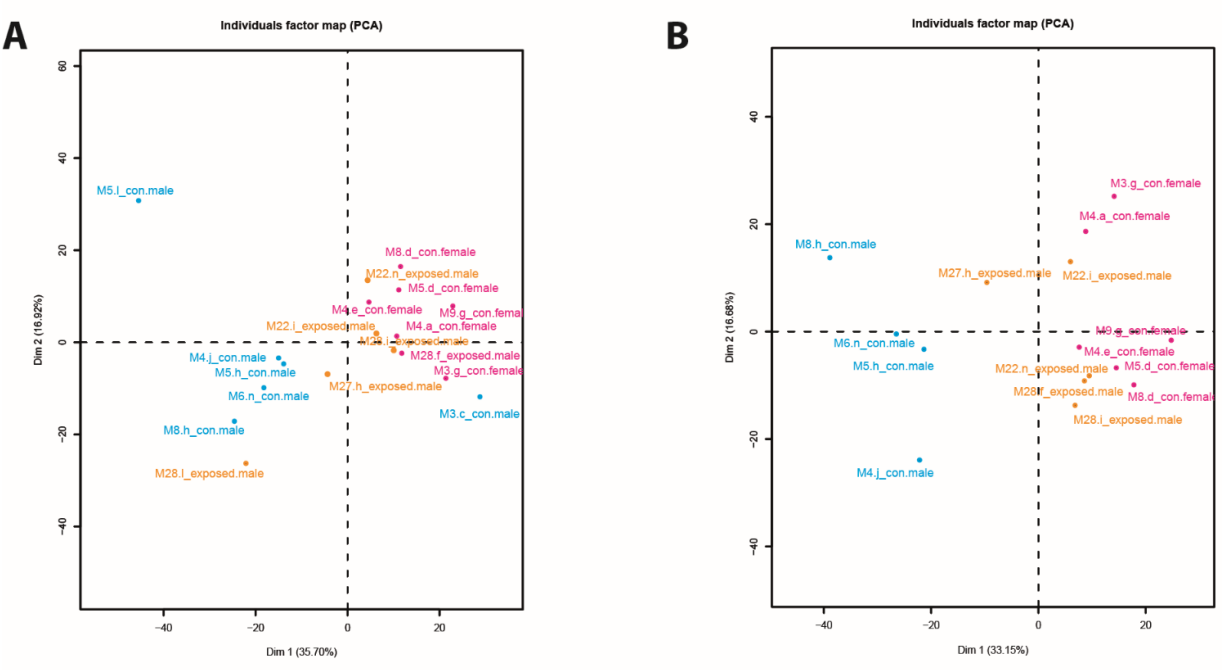

Figure S3. Quality control of gene array data. A: The PCA plot allowed us to identify three outliers that were subsequently removed from the analysis. **B:** PCA plot in which the outliers have been excluded.

Supplementary Table 1

Supplementary Table S1 - Pregnancy and litter data

Data represent group means, based on litter means ± SD after oral exposure GD7-21.
FIN= Finasteride, 10 mg/kg bw/day

	Control	FIN
GD21 Caesarean section		
No. of litters	N=6	N=6
Maternal body weight (g), GD 7	262.2 ± 12.2	277.3 ± 16.6
No. of fetuses	14.0 ± 2.4	12.5 ± 2.4
Maternal bw gain GD7- 21 (g)	115.3 ± 18.8	109.3 ± 7.4
Adjusted maternal bw (g)^	289.7 ± 20.0	313.2 ± 14.5
Uterus weight (g)	87.8 ± 10.8	73.4 ± 13.6
Fetal weight male (g) (bw)	4.3 ± 0.4	**3.9 ± 0.3***
Fetal weight female (g) (bw)	4.2 ± 0.3	**3.6 ± 0.5**$^{¤}$
Male AGD (mm)	3.64 ± 0.2	**2.28 ± 0.1****
Male AGD index	2.24 ± 0.03	**1.45 ± 0.08***
Cryptorchidism male fetuses	0	5$^{\#}$
Female AGD (mm)	1.81 ± 0.09	1.70 ± 0.11
Female AGD index	1.13 ± 0.04	1.11 ± 0.04

This table covers the data presented in Table 1 supplemented with data from the dams and fetuses
Values in **bold** are statistically different from Control
* Significantly different from Control, $p < 0.05$
** Significantly different from Control, $p < 0.01$
$^{\#}$ One litter had three cryptorchid males while two litters had one cryptorchid male, p=0.062 (Fisher's Exact test)
$^{¤}$ Tends to be different from control, $p = 0.055$
^ Adjusted maternal bw (g) is weight GD 21 after subtracting the uterus weight
AGD (mm) is analysed with fetal weight as covariate.
AGD index = AGD divided by cube root of the bodyweight

Supplementary Table 2

Supplementary table 2 is too elaborate to be included here. Please contact the author to have the file send by email or find it at https://doi.org/10.1093/toxsci/kfz046

Supplementary Table 3

Compared to control males

Elements only upregulated in control female when compared to control male (91)

Fsd2	Mapk12	Ppp1r27	Popdc3	Arpp19	Atp2b3	Dusp26	Ppargc1a
Thbs4	Col8a1	Usp13	Mir431	Cacna2d1	Ptp4a3	Prob1	Ppp1r1a
Jsrp1	Macrod1	Fam78a	Phospho1	Ablim3	Ehbp1l1	Dmpk	Hbegf
LOC10036(	Slc29a4	LOC10369:	Ldhb	P2rx5	Epdr1	Rragd	Speg
Rab40b	Slc6a13	Efr3b	Snta1	Ebf2	Camk2a	Cdk5r1	Inhba
Myadml2	Ctnna3	Vstm2a	Ttll7	Scn3b	Reep1	Mybph	Akap6
Gpr155	Phex	Chrng	Stra6	Got1	Fgf13	Ppp1r9a	Acacb
Ache	Mum1l1	Tpm1	Megf10	Slc25a12	Cfl2	Fgfrl1	Atp9a
Fndc5	Fxyd1	Mamstr	Chrna1	Mapt	Itga7	Slc26a7	Mgp
Pcbd1	Rrad	Ryr3	Hspb8	Sec1	Krt16	Kcnma1	Usp2
Dcx	Ramp1	Cdh15	Gamt	Shisa4	Schip1	Ky	Zbtb18
Prokr2	Fgf1	Tatdn3					

Elements only upregulated in exposed males when compared to control male (5)

Pnoc	Cxcl13	Myh7b	Lcn2	LOC102556346

Elements upregulated in both control female and exposed male when compared to control male (222)

Myl3	Rxfp2	Myl2	Ckmt2	Nrap	Myh7	Art3	Casq1
Hspb7	Myoz1	Atp1b4	Tnni2	Tmem182	Sypl2	Mylk2	Coro6
Mlip	Dtna	Atp2a1	Apobec2	Ldb3	Sln	Cryab	Tmod1
Cox8b	Pygm	Myf6	Casq2	Mlf1	Cpt1b	Cav3	Mypn
LOC29813!	Alpk3	Tnnt3	Klhl41	Mustn1	Perm1	RGD15648!	Myo18b
Fitm1	Gatm	Cacnb1	Tnnc1	Synpo2l	Ankrd23	LOC10255!	Chodl
Tnnt1	Myl4	Myh8	Adprhl1	Mchr1	Ak1	Rps2-ps7	Asb5
Des	Hspb2	Cilp	Asb4	Gmpr	Cacng6	Pkia	Ppara
Chrnd	Prkaa2	Sgca	Ptges3l	Best3	Coq8a	Eda2r	Cpa1
Prepl	Ip6k3	Gstm7	Myl1	Acta1	Tceal5	Ppm1e	Mir410
Zim1	Lmo3	Smtnl2	Aldh1a2	Chrnb1	Abca8a	Klhl38	Mef2c
Cavin4	Cmya5	Trim72	Ampd1	Tmod4	Myoz2	Trdn	Lrrc39
Myom1	Tceal7	Actn3	Pkm	Cox6a2	Tecrl	Xirp1	Smtnl1
Itgb1bp2	Smpx	Cacna1s	Lmod3	Tnni1	Lsmem2	Pgam2	Esr1
Lmcd1	Cacng1	Sgcg	Sh3bgr	Pdk4	Srl	Hfe2	Mb
Hrc	Mybpc1	Fabp3	Map3k7cl	Ankrd2	Actn2	Pde4dip	Ttn
Capn3	Zdbf2	Trim63	Lsmem1	Ryr1	Igsf1	Agl	Adssl1
Padi2	LOC10254!	Tnnc2	Popdc2	Mymk	Smyd1	Sfrp4	Cap2
Mylpf	Vgll2	Chchd10	Tmem38b	Itgb6	Stac3	Hspb3	Myh3
Jph2	Pdlim3	Pgm1	Pfkm	Dok5	Tm6sf1	LOC100911	Arpp21
Hspb6	Myog	Srpk3	Ppp2r3a	Plpp7	Actc1	Trim55	Myf5
Prr32	Tpm2	Iffo1	Sntb1	Cidea	Musk	Bin1	Sspn
Myom2	Xirp2	Eef1a2	Ppp1r3a	Csrp3	LOC10835(	Nexn	Slc16a3
Yipf7	Arhgap36	Klhl31	Myom3	Tmem38a	Unc45b	Tnnt2	Dmd
Txlnb	Ckm	Rbfox1	Sel1l3	Trim54	Stbd1	Asb18	Obscn
Pstpip2	Asb2	Eno3	Fndc3c1	Neb	Slc16a6	Rtn2	Bves
Dhrs7c	Sptb	Filip1l	Art1	Camk2b	Rbm24	Flnc	Jph1
Ankrd1	Dusp27	Fhl1	Hacd1	Pacsin3	Klhl40		

Elements only downregulated in control female when compared to control male (29)

Ddx3	Eif2s3y	Cidec	Retn	Cyp1b1	Ca5b	Smoc1	Vegfd
LOC10091:	Srd5a2	Wnt2	Slc5a7	Adgrd1	Cbln2	Irs4	Olr522
Cacnb2	Ccl22	Loxl4	Medag	Dpep1	Il33	Aff2	Ltbp2
RGD15652!	Lgals12	Papss2	Gabra2	Hmg1l1			

Elements only downregulated in exposed male when compared to control male (8)

Rpl10a	Sprr1b	Pnlip	Ceacam1	Fxyd3	Myocd	Fam107a	Stfa2l1

Elements downregulated in both control female and exposed male when compared to control male (6)

Adamts16	Igfbp2	Pappa2	Dhrs2	Gria3	Stmn4

Compared to control females

Elements only upregulated in control male when compared to control female (33)

Cidec	Adamts16	Retn	Cyp1b1	Ca5b	Igfbp2	Smoc1	Pappa2
Lgals12	Papss2	Gabra2	Hmg1l1	Cacnb2	Ccl22	Loxl4	Medag
Vegfd	Dhrs2	LOC100911	Gria3	Srd5a2	Wnt2	Slc5a7	Adgrd1
Dpep1	Il33	Aff2	Ltbp2	Cbln2	Irs4	Stmn4	Olr522
RGD1565297							

Elements only upregulated in exposed male when compared to control female (2)

Pnoc LOC100910135

Elements upregulated in both control male and exposed male when compared to control female (2)

Ddx3 Eif2s3y

Elements only downregulated in control male when compared to control female (307)

Myl3	Rxfp2	Myl2	Ckmt2	Nrap	Myh7	Art3	Casq1
Hspb7	Myoz1	Atp1b4	Tnni2	Tmem182	Sypl2	Mylk2	Coro6
Atp2a1	Apobec2	Ldb3	Sln	Cryab	Tmod1	Itgb1bp2	Smpx
Casq2	Mlf1	Cpt1b	Cav3	Fsd2	Mypn	Lmcd1	Cacng1
Tnnt3	Klhl41	Mustn1	Perm1	RGD15648	Myo18b	Hrc	Mybpc1
Tnnc1	Synpo2l	Ankrd23	LOC102555	Chodl	Capn3	Zdbf2	Trim63
Tnnt1	Myl4	Myh8	Adprhl1	Mchr1	Ak1	Rps2-ps7	Asb5
Klhl40	Ctnna3	Stbd1	Asb18	Obscn	Des	Hspb2	Cilp
Myh3	Fhl1	Hacd1	Slc16a6	Rtn2	Bves	Chrnd	Prkaa2
Arpp21	Nexn	Slc16a3	Ttll7	Rbm24	Scn3b	Reep1	Flnc
Mir410	Hspb6	Myog	Srpk3	Ppp2r3a	Hspb8	Plpp7	Actc1
Sspn	LOC10835(	Gpr155	Phex	Chrng	Stra6	Got1	Zim1
Prr32	Gamt	Shisa4	Jsrp1	Macrod1	Fam78a	Phospho1	Tpm2
Prokr2	Schip1	Ky	Zbtb18	Fgf1	LOC10036(	Slc29a4	Ppm1e
Mgp	Tatdn3	Krt16	Kcnma1	Usp2	Inhba	Rab40b	Slc6a13
Cavin4	Cmya5	Trim72	Ampd1	Tmod4	Myoz2	Trdn	Lrrc39
Myom1	Tceal7	Pkm	Cox6a2	Tecrl	Xirp1	Smtnl1	Yipf7
Cacna1s	Lmod3	Tnni1	Lsmem2	Pgam2	Esr1	Txlnb	Ckm
Sgcg	Sh3bgr	Pdk4	Srl	Hfe2	Mb	Pstpip2	Asb2
Fabp3	Ankrd2	Actn2	Pde4dip	Ttn	Dhrs7c	Sptb	Filip1l
Lsmem1	Ryr1	Mapk12	Igsf1	Agl	Adssl1	Ankrd1	Ppp1r27
Arpp19	Padi2	LOC10254	Tnnc2	Popdc2	Mymk	Smyd1	Sfrp4
Asb4	Gmpr	Cacng6	Pkia	Ppara	Mylpf	Vgll2	Chchd10
Sgca	Ptges3l	Best3	Coq8a	Eda2r	Cpa1	Jph2	Pdlim3
Jph1	Mybph	Akap6	Prepl	Ip6k3	Gstm7	Pcbd1	Rrad
Trim55	Myf5	Sec1	Thbs4	Cidea	Usp13	Mir431	Musk
Lmo3	Smtnl2	Aldh1a2	Fgf13	Ppp1r9a	Chrnb1	Abca8a	Klhl38
Ablim3	Ehbp1l1	Dmpk	Hbegf	Iffo1	Ache	Mum1l1	Tpm1
Ldhb	P2rx5	Epdr1	Rragd	Speg	Fndc5	Sntb1	Fxyd1
Efr3b	Snta1	Ebf2	Camk2a	Cdk5r1	Stac3	Vstm2a	Hspb3
Myom2	Xirp2	Eef1a2	Ppp1r3a	Csrp3	Pgm1	Pfkm	Dok5
Arhgap36	Klhl31	Tmem38a	Mlip	Dtna	Myl1	Acta1	Tceal5
Rbfox1	Trim54	Cox8b	Pygm	Myf6	Bin1	Cacna2d1	Ptp4a3
Eno3	Fndc3c1	Neb	LOC29813	Alpk3	Acacb	Dcx	Mef2c
Art1	Camk2b	Fitm1	Gatm	Cacnb1	Megf10	Slc25a12	Cfl2
Dusp27	Unc45b	Popdc3	Tnnt2	Dmd	Mamstr	Chrna1	Mapt
Cap2	Dusp26	Pacsin3	Ppargc1a	Myadml2	Tm6sf1	LOC100912	Ryr3
Tmem38b	Itgb6	Prob1	Ppp1r1a	Itga7	Slc26a7	Fgfrl1	Atp9a
Ramp1	Cdh15	LOC103693936					

Elements only downregulated in exposed male when compared to control female (2)

Cpa6 Lce3e

Elements downregulated in both control male and exposed male when compared to control female (6)

Actn3	Myom3	Sel1l3	Map3k7cl	Atp2b3	Col8a1

Supplementary Table 4

Cluster	Category	Term	Entry	r	R	n	N	rEXP	r/R	p(z)	z	p	p(hommel)	p(BH)
Cluster 1	biological_process	0006936 muscle contraction	114207/Ryr1;117505/Csrp3;117558/Myll	52	272	195	17276	3,070155	0,191176	1,42E-176	28,30689	1,42E-49	5,49E-46	1,83E-46
Cluster 1	biological_process	0006941 striated musde contraction	117505/Csrp3;117558/Mylk2;170546/Ryr	41	272	116	17276	1,826349	0,150735	3,28E-189	29,31559	8,04E-45	3,10E-41	6,20E-42
Cluster 1	biological_process	0007519 skeletal muscle tissue development	113995/P2rx5;114108/Pdlim3;114207/Ry	35	272	155	17276	2,44038	0,128676	3,74E-99	21,10285	1,15E-30	4,40E-27	3,40E-28
Cluster 1	biological_process	0030239 myofibril assembly	100910104/Xirp1;117505/Csrp3;117537/	24	272	57	17276	0,89743	0,088235	3,74E-134	24,62131	9,14E-29	3,51E-25	2,52E-26
Cluster 1	biological_process	0003009 skeletal muscle contraction	171409/Tnnt1;24261/Chrnb1;24583/Myl	17	272	35	17276	0,551053	0,0625	5,15E-111	22,35708	4,78E-22	1,84E-18	1,02E-19
Cluster 1	biological_process	0060048 cardiac muscle contraction	117505/Csrp3;245585/Myl3;245956/Scn3	19	272	72	17276	1,133596	0,069853	9,76E-65	16,94917	1,65E-18	6,33E-15	2,55E-16
Cluster 1	biological_process	0045214 sarcomere organization	100910104/Xirp1;117537/Klhl41;24837/I	15	272	38	17276	0,598287	0,055147	4,77E-79	18,78759	6,89E-18	2,64E-14	9,83E-16
Cluster 1	biological_process	0006937 regulation of muscle contraction	116601/Atp2a1;117558/Mylk2;171009/A	24	272	151	17276	2,377402	0,088235	4,78E-46	14,19699	1,51E-17	5,80E-14	2,09E-15
Cluster 1	biological_process	0048747 muscle fiber development	114207/Ryr1;117537/Klhl41;24261/Chrn	16	272	55	17276	0,865941	0,058824	7,03E-61	16,41864	1,57E-16	6,02E-13	2,02E-14
Cluster 1	biological_process	0048513 animal organ development	100910104/Xirp1;113995/P2rx5;114108/	104	272	3276	17276	51,57861	0,382353	1,51E-16	8,172686	6,45E-14	2,47E-10	7,11E-12
Cluster 1	biological_process	0055013 cardiac muscle cell development	100910104/Xirp1;117505/Csrp3;246172/	14	272	61	17276	0,960408	0,051471	1,89E-41	13,435	4,79E-13	1,83E-09	4,86E-11
Cluster 1	biological_process	0010830 regulation of myotube differentiation	117505/Csrp3;171009/Actn3;25714/Myf	14	272	61	17276	0,960408	0,051471	1,89E-41	13,435	4,79E-13	1,83E-09	4,86E-11
Cluster 1	biological_process	0014819 regulation of skeletal muscle contraction	116601/Atp2a1;117558/Mylk2;171009/A	8	272	11	17276	0,173188	0,029412	1,74E-80	18,96258	5,40E-13	2,06E-09	5,34E-11
Cluster 1	biological_process	0014902 myotube differentiation	114207/Ryr1;117537/Klhl41;24907/Dmd	14	272	63	17276	0,991896	0,051471	5,08E-40	13,18887	7,73E-13	2,95E-09	7,45E-11
Cluster 1	biological_process	0044763 single-organism cellular process	100910104/Xirp1;113995/P2rx5;114108/	185	272	8361	17276	131,6388	0,680147	3,38E-11	6,525713	3,52E-11	1,34E-07	2,71E-09
Cluster 1	biological_process	0042391 regulation of membrane potential	100910104/Xirp1;113995/P2rx5;24261/C	29	272	429	17276	6,754341	0,106618	1,20E-18	8,736703	4,25E-11	1,62E-07	3,21E-09
Cluster 1	biological_process	0035914 skeletal muscle cell differentiation	117537/Klhl41;117558/Mylk2;25714/My	12	272	60	17276	0,944663	0,044118	7,87E-31	11,48477	1,30E-10	4,94E-07	9,47E-09
Cluster 1	biological_process	0055008 cardiac muscle tissue morphogenesis	117558/Mylk2;24585/Myl3;24837/Tnnt2	12	272	64	17276	1,007641	0,044118	1,00E-28	11,05802	2,89E-10	1,09E-06	1,95E-08
Cluster 1	biological_process	0007600 sensory perception	245956/Scn3b;25516/Pnoc;83731/Kcnm	3	272	1983	17276	31,22112	0,011029	1	-5,410765	1	0,999999904	1
Cluster 1	biological_process	0014874 response to stimulus involved in regulation of muscle adaptation	140939/Trim63;171009/Actn3;24907/Dm	8	272	20	17276	0,314888	0,029412	1,08E-43	13,81203	3,64E-10	1,38E-06	2,38E-08
Cluster 1	biological_process	0014850 response to musde activity	260327/Fndc5;29148/Myog;29155/Capn	9	272	29	17276	0,456587	0,033088	1,47E-37	12,75463	3,96E-10	1,50E-06	2,55E-08
Cluster 1	biological_process	0010880 regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	24907/Dmd;287411/Dhrs7c;29209/Casq:	8	272	21	17276	0,330632	0,029412	1,50E-41	13,45193	5,81E-10	2,20E-06	3,55E-08
Cluster 1	biological_process	1901019 regulation of calcium ion transmembrane transporter activity	24907/Dmd;25399/Cacna2d1;29161/Cav	13	272	85	17276	1,338273	0,047794	1,15E-24	10,1858	7,38E-10	2,79E-06	4,38E-08
Cluster 1	biological_process	0060314 regulation of ryanodine-sensitive calcium-release channel activity	24907/Dmd;29209/Casq2;292905/Hrc;2	8	272	22	17276	0,346376	0,029412	1,33E-39	13,11605	9,00E-10	3,40E-06	5,26E-08
Cluster 1	biological_process	0048641 regulation of skeletal muscle tissue development	115771/Usp2;117537/Klhl41;171009/Act	11	272	57	17276	0,89743	0,040441	2,47E-27	10,7667	1,17E-09	4,42E-06	6,54E-08
Cluster 1	biological_process	0010831 positive regulation of myotube differentiation	117505/Csrp3;171009/Actn3;25714/Myf	9	272	34	17276	0,535309	0,033088	8,79E-32	11,67267	1,94E-09	7,30E-06	1,05E-07
Cluster 1	biological_process	0048646 anatomical structure formation involved in morphogenesis	100910104/Xirp1;116671/Cdk5r1;11750	39	272	855	17276	13,46145	0,143382	3,09E-13	7,19624	2,12E-09	7,98E-06	1,12E-07

Cluster	Category	Term	Entry	r	R	n	N	rEXP	r/R	p(z)	z	p	p(hommel)	p(BH)
Cluster 1	biological_process	0032879 regulation of localization	114207/Ryr1;114906/Pkia;116671/Cdk5r	80	272	2659	17276	41,86432	0,294118	5,29E-11	6,458556	2,69E-09	1,02E-05	1,37E-07
Cluster 1	biological_process	0010959 regulation of metal ion transport	245956/Scn3b;24907/Dmd;25177/Fhl1;2	25	272	401	17276	6,313498	0,091912	1,67E-14	7,584486	5,25E-09	1,98E-05	2,56E-07
Cluster 1	biological_process	0048468 cell development	100910104/Xirp1;114207/Ryr1;116671/(	57	272	1625	17276	25,58463	0,209559	2,40E-11	6,577135	5,47E-09	2,06E-05	2,64E-07
Cluster 1	biological_process	0065008 regulation of biological quality	100910104/Xirp1;113995/P2rx5;114207/	95	272	3473	17276	54,68025	0,349265	3,91E-10	6,148525	6,71E-09	2,52E-05	3,09E-07
Cluster 1	biological_process	0051239 regulation of multicellular organismal process	113995/P2rx5;115771/Usp2;116601/Atp	82	272	2811	17276	44,25747	0,301471	2,06E-10	6,249332	6,73E-09	2,53E-05	3,09E-07
Cluster 1	biological_process	0048741 skeletal muscle fiber development	114207/Ryr1;117537/Klhl41;29148/Myo	8	272	28	17276	0,440843	0,029412	7,88E-31	11,48464	8,06E-09	3,03E-05	3,61E-07
Cluster 1	biological_process	0032989 cellular component morphogenesis	100910104/Xirp1;116653/Cap2;116671/(	35	272	766	17276	12,0602	0,128676	4,86E-12	6,810734	1,49E-08	5,57E-05	6,28E-07
Cluster 1	biological_process	0033275 actin-myosin filament sliding	24837/Tnnt2;24851/Tpm1;252942/Myh8	5	272	7	17276	0,110211	0,018382	3,53E-50	14,84909	1,91E-08	7,16E-05	7,91E-07
Cluster 1	biological_process	0002027 regulation of heart rate	245956/Scn3b;24851/Tpm1;24907/Dmd;	12	272	92	17276	1,448483	0,044118	3,99E-19	8,860354	2,16E-08	8,09E-05	8,86E-07
Cluster 1	biological_process	1903115 regulation of actin filament-based movement	117558/Mylk2;24837/Tnnt2;290561/Tnn	9	272	44	17276	0,692753	0,033088	3,64E-24	10,07291	2,28E-08	8,55E-05	9,26E-07
Cluster 1	biological_process	0014888 striated muscle adaptation	140939/Trim63;171009/Actn3;29148/My	8	272	32	17276	0,50382	0,029412	8,30E-27	10,65463	2,58E-08	9,68E-05	1,04E-06
Cluster 1	biological_process	0014894 response to denervation involved in regulation of muscle adaptation	140939/Trim63;171009/Actn3;24907/Dm	6	272	14	17276	0,220421	0,022059	1,11E-35	12,41306	3,89E-08	0,000145688	1,49E-06
Cluster 1	biological_process	0043501 skeletal muscle adaptation	140939/Trim63;171009/Actn3;29148/My	6	272	14	17276	0,220421	0,022059	1,11E-35	12,41306	3,89E-08	0,000145688	1,49E-06
Cluster 1	biological_process	0014866 skeletal myofibril assembly	25566/Tmod1;29275/Actc1;29437/Acta1	5	272	8	17276	0,125955	0,018382	6,75E-44	13,84573	5,02E-08	0,000187829	1,86E-06
Cluster 1	biological_process	0048743 positive regulation of skeletal muscle fiber development	171009/Actn3;25714/Myf6;29148/Myog	5	272	8	17276	0,125955	0,018382	6,75E-44	13,84573	5,02E-08	0,000187829	1,86E-06
Cluster 1	biological_process	0007015 actin filament organization	114108/Pdlim3;116653/Cap2;24837/Tnn	16	272	195	17276	3,070155	0,058824	3,71E-14	7,480172	8,21E-08	0,000306061	2,90E-06
Cluster 1	biological_process	0070252 actin-mediated cell contraction	245956/Scn3b;24837/Tnnt2;24851/Tpm1	9	272	51	17276	0,802964	0,033088	1,30E-20	9,233883	8,89E-08	0,000331475	3,09E-06
Cluster 1	biological_process	0051606 detection of stimulus	117505/Csrp3;29161/Cav3;84015/Ttn	3	272	1633	17276	25,71058	0,011029	0,999999	-4,744248	1	0,999974713	0,999997
Cluster 1	biological_process	0048643 positive regulation of skeletal muscle tissue development	115771/Usp2;171009/Actn3;25714/Myf6	7	272	27	17276	0,425098	0,025735	1,32E-24	10,17226	1,51E-07	0,000560509	5,09E-06
Cluster 1	biological_process	2001257 regulation of cation channel activity	24907/Dmd;25399/Cacna2d1;291245/Ac	14	272	157	17276	2,471868	0,051471	5,66E-14	7,424413	1,93E-07	0,000717778	6,47E-06
Cluster 1	biological_process	0051049 regulation of transport	114906/Pkia;116671/Cdk5r1;117028/Bin	60	272	1944	17276	30,60708	0,220588	6,56E-09	5,684436	2,07E-07	0,000770665	6,89E-06
Cluster 1	biological_process	0014883 transition between fast and slow fiber	171009/Actn3;171409/Tnnt1;290561/Tn	5	272	10	17276	0,157444	0,018382	4,27E-35	12,3047	2,20E-07	0,000818551	7,20E-06
Cluster 1	biological_process	0071688 striated muscle myosin thick filament assembly	25566/Tmod1;306616/Myom2;362867/M	5	272	10	17276	0,157444	0,018382	4,27E-35	12,3047	2,20E-07	0,000818551	7,20E-06
Cluster 1	biological_process	0045661 regulation of myoblast differentiation	117505/Csrp3;117537/Klhl41;25714/Myf	9	272	59	17276	0,928919	0,033088	1,39E-17	8,455109	3,29E-07	0,001218657	1,05E-05
Cluster 1	biological_process	0030154 cell differentiation	100910104/Xirp1;114207/Ryr1;116671/(	89	272	3445	17276	54,23941	0,327206	5,28E-08	5,316894	3,93E-07	0,001453485	1,24E-05
Cluster 1	biological_process	0070296 sarcoplasmic reticulum calcium ion transport	114207/Ryr1;116601/Atp2a1;29658/Cacı	5	272	11	17276	0,173188	0,018382	6,82E-32	11,69427	3,99E-07	0,001474995	1,24E-05
Cluster 1	biological_process	0071313 cellular response to caffeine	114207/Ryr1;29209/Casq2;682930/Cacn	5	272	11	17276	0,173188	0,018382	6,82E-32	11,69427	3,99E-07	0,001474995	1,24E-05
Cluster 1	biological_process	0006816 calcium ion transport	114207/Ryr1;116601/Atp2a1;140727/Ca	16	272	222	17276	3,495254	0,058824	5,79E-12	6,785432	4,84E-07	0,001787169	1,48E-05
Cluster 1	biological_process	0006874 cellular calcium ion homeostasis	114207/Ryr1;116601/Atp2a1;117505/Csı	22	272	406	17276	6,39222	0,080882	1,52E-10	6,296697	5,14E-07	0,001895908	1,56E-05
Cluster 1	biological_process	0043403 skeletal muscle tissue regeneration	24907/Dmd;25438/Eno3;25630/Pkm;257	7	272	33	17276	0,519565	0,025735	5,92E-20	9,070537	6,68E-07	0,002463763	1,99E-05

Cluster	Category	Term	Entry	r	R	n	N	rEXP	r/R	p(z)	z	p	p(hommel)	p(BH)
Cluster 1	biological_process	0035994 response to muscle stretch	117505/Csrp3;24907/Dmd;27064/Ankrd2		6	272	24	17276	0,377865	0,022059	1,42E-20	9,225037	1,53E-06	0,005613937	4,12E-05
Cluster 1	biological_process	0042246 tissue regeneration	24907/Dmd;25438/Eno3;25630/Pkm;257		9	272	72	17276	1,133596	0,033088	4,24E-14	7,462555	1,86E-06	0,006836444	4,96E-05
Cluster 1	biological_process	0045663 positive regulation of myoblast differentiation	117505/Csrp3;25714/Myf6;29148/Myog		6	272	26	17276	0,409354	0,022059	6,04E-19	8,813999	2,55E-06	0,009326527	6,68E-05
Cluster 1	biological_process	0010469 regulation of receptor activity	24907/Dmd;291245/Actn2;29161/Cav3;2		12	272	145	17276	2,282936	0,044118	3,77E-11	6,509564	3,21E-06	0,011749364	8,31E-05
Cluster 1	biological_process	0014889 muscle atrophy	140939/Trim63;171009/Actn3;29148/My		5	272	16	17276	0,25191	0,018382	7,17E-22	9,539613	3,53E-06	0,012927311	8,97E-05
Cluster 1	biological_process	1901077 regulation of relaxation of muscle	171009/Actn3;292905/Hrc;367086/Sln;6		5	272	16	17276	0,25191	0,018382	7,17E-22	9,539613	3,53E-06	0,012927311	8,97E-05
Cluster 1	biological_process	0007274 neuromuscular synaptic transmission	117558/Mylk2;24261/Chrnb1;25753/Chr		6	272	31	17276	0,488076	0,022059	8,64E-16	7,959449	7,62E-06	0,027628901	0,000175
Cluster 1	biological_process	0051196 regulation of coenzyme metabolic process	171009/Actn3;24959/Pgam2;25747/Ppar		7	272	47	17276	0,739986	0,025735	1,03E-13	7,344945	8,15E-06	0,029519246	0,000185
Cluster 1	biological_process	0003231 cardiac ventricle development	24585/Myl3;24837/Tnnt2;24851/Tpm1;2		11	272	133	17276	2,094003	0,040441	2,37E-10	6,227382	8,33E-06	0,03015612	0,000187
Cluster 1	biological_process	0009887 animal organ morphogenesis	114207/Ryr1;116676/Aldh1a2;117558/My		34	272	962	17276	15,1461	0,125	2,52E-07	5,02488	8,60E-06	0,031149626	0,000191
Cluster 1	biological_process	1902903 regulation of supramolecular fiber organization	24851/Tpm1;25420/Cryab;25566/Tmod1		17	272	309	17276	4,865015	0,0625	1,10E-08	5,595639	8,58E-06	0,031061484	0,000191
Cluster 1	biological_process	0055117 regulation of cardiac muscle contraction	24907/Dmd;29161/Cav3;29209/Casq2;29		8	272	66	17276	1,039129	0,029412	2,68E-12	6,895938	8,88E-06	0,032134174	0,000196
Cluster 1	biological_process	0046716 muscle cell cellular homeostasis	24907/Dmd;29161/Cav3;366624/Cfl2;65		5	272	19	17276	0,299143	0,018382	2,20E-18	8,667818	9,05E-06	0,032743772	0,000198
Cluster 1	biological_process	0055010 ventricular cardiac muscle tissue morphogenesis	24585/Myl3;24837/Tnnt2;24851/Tpm1;2		7	272	49	17276	0,771475	0,025735	4,10E-13	7,157718	1,08E-05	0,039142743	0,000231
Cluster 1	biological_process	0030834 regulation of actin filament depolymerization	24851/Tpm1;25566/Tmod1;291245/Actn		7	272	51	17276	0,802964	0,025735	1,47E-12	6,980902	1,42E-05	0,051245335	0,000293
Cluster 1	biological_process	0007186 G-protein coupled receptor signaling pathway	116671/Cdk5r1;192649/Prokr2;24890/Es		12	272	2292	17276	36,08613	0,044118	0,999993	-4,339484	0,999999	0,996850845	0,999649
Cluster 1	biological_process	0048589 developmental growth	113995/P2rx5;116671/Cdk5r1;24890/Esr		20	272	433	17276	6,817319	0,073529	1,28E-07	5,153975	1,85E-05	0,066310379	0,000371
Cluster 1	biological_process	0086004 regulation of cardiac muscle cell contraction	29161/Cav3;29209/Casq2;361839/Ctnna		6	272	37	17276	0,582542	0,022059	3,98E-13	7,161943	2,22E-05	0,079748532	0,000444
Cluster 1	biological_process	0044724 single-organism carbohydrate catabolic process	24645/Pgm1;24701/Pygm;24959/Pgam2		8	272	76	17276	1,196573	0,029412	1,66E-10	6,282738	2,55E-05	0,091117759	0,000498
Cluster 1	biological_process	0001508 action potential	113995/P2rx5;245956/Scn3b;24907/Dmd		9	272	99	17276	1,558694	0,033088	8,46E-10	6,024904	2,62E-05	0,093727699	0,000507
Cluster 1	biological_process	1902904 negative regulation of supramolecular fiber organization	24851/Tpm1;25420/Cryab;25566/Tmod1		10	272	126	17276	1,983793	0,036765	4,27E-09	5,757631	3,07E-05	0,109703027	0,00059
Cluster 1	biological_process	0003206 cardiac chamber morphogenesis	24585/Myl3;24837/Tnnt2;24851/Tpm1;2		10	272	127	17276	1,999537	0,036765	5,21E-09	5,723822	3,29E-05	0,117287316	0,000628
Cluster 1	biological_process	0006812 cation transport	113995/P2rx5;114207/Ryr1;116601/Atp2		29	272	814	17276	12,81593	0,106618	1,52E-06	4,667953	3,59E-05	0,127806807	0,000674
Cluster 1	biological_process	0006091 generation of precursor metabolites and energy	24645/Pgm1;24701/Pygm;24959/Pgam2		14	272	253	17276	3,983329	0,051471	1,73E-07	5,096092	5,05E-05	0,178332113	0,000928
Cluster 1	biological_process	0007271 synaptic transmission, cholinergic	24261/Chrnb1;25753/Chrng;54240/Chrn		5	272	27	17276	0,425098	0,018382	7,31E-13	7,077993	5,67E-05	0,199331408	0,001036
Cluster 1	biological_process	0034220 ion transmembrane transport	113995/P2rx5;114207/Ryr1;116601/Atp2		28	272	796	17276	12,53253	0,102941	3,26E-06	4,50896	6,15E-05	0,21585707	0,001113
Cluster 1	biological_process	1901880 negative regulation of protein depolymerization	24851/Tpm1;25566/Tmod1;295261/Tmo		7	272	64	17276	1,007641	0,025735	8,29E-10	6,028155	6,41E-05	0,224801515	0,001155
Cluster 1	biological_process	0007528 neuromuscular junction development	315962/Ky;362242/Snta1;50688/Cacnb1		6	272	45	17276	0,708497	0,022059	1,11E-10	6,34468	7,01E-05	0,245276898	0,001258
Cluster 1	biological_process	0070588 calcium ion transmembrane transport	114207/Ryr1;116601/Atp2a1;140727/Ca		11	272	168	17276	2,645057	0,040441	9,79E-08	5,203331	7,34E-05	0,256374192	0,001304
Cluster 1	biological_process	0051926 negative regulation of calcium ion transport	29161/Cav3;29209/Casq2;292905/Hrc;31		7	272	66	17276	1,039129	0,025735	1,76E-09	5,905267	7,82E-05	0,272505736	0,001378
Cluster 1	biological_process	0048729 tissue morphogenesis	116676/Aldh1a2;117558/Mylk2;24585/My		23	272	599	17276	9,430887	0,084559	2,91E-06	4,53285	7,92E-05	0,275652962	0,001388

Cluster	Category	Term	Entry	r	R	n	N	rEXP	r/R	p(z)	z	p	p(hommel)	p(BH)
Cluster 1	biological_process	0005977 glycogen metabolic process	24645/Pgm1;24701/Pygm;305234/Stbd1	6	272	48	17276	0,75573	0,022059	5,68E-10	6,088905	0,000102	0,348937951	0,00174
Cluster 1	biological_process	0046034 ATP metabolic process	24183/Ak1;24583/Myh3;24959/Pgam2;2	11	272	176	17276	2,771012	0,040441	2,75E-07	5,00823	0,000111	0,380018726	0,001899
Cluster 1	biological_process	0051693 actin filament capping	24851/Tpm1;25566/Tmod1;295261/Tmo	5	272	31	17276	0,488076	0,018382	3,62E-11	6,515407	0,000113	0,386251743	0,001916
Cluster 1	biological_process	0002026 regulation of the force of heart contraction	117505/Csrp3;24585/Myl3;29557/Myh7;	5	272	31	17276	0,488076	0,018382	3,62E-11	6,515407	0,000113	0,386251743	0,001916
Cluster 1	biological_process	0051480 regulation of cytosolic calcium ion concentration	114207/Ryr1;170546/Ryr3;24890/Esr1;2	15	272	308	17276	4,849271	0,055147	1,38E-06	4,688121	0,000116	0,394686485	0,001945
Cluster 1	biological_process	1901021 positive regulation of calcium ion transmembrane transporter activity	25399/Cacna2d1;296345/Jph2;59299/Tr	5	272	32	17276	0,50382	0,018382	8,26E-11	6,390604	0,000133	0,445040305	0,002185
Cluster 1	biological_process	0046320 regulation of fatty acid oxidation	116719/Acacb;25747/Ppara;79131/Fabp	5	272	32	17276	0,50382	0,018382	8,26E-11	6,390604	0,000133	0,445040305	0,002185
Cluster 1	biological_process	0060306 regulation of membrane repolarization	25399/Cacna2d1;29161/Cav3;29209/Cas	5	272	32	17276	0,50382	0,018382	8,26E-11	6,390604	0,000133	0,445040305	0,002185
Cluster 1	biological_process	0043462 regulation of ATPase activity	24837/Tnnt2;24838/Tnnt3;24851/Tpm1;	7	272	73	17276	1,14934	0,025735	1,77E-08	5,512309	0,000149	0,494015943	0,002425
Cluster 1	biological_process	0060079 excitatory postsynaptic potential	113995/P2rx5;24261/Chrnb1;25753/Chr	7	272	73	17276	1,14934	0,025735	1,77E-08	5,512309	0,000149	0,494015943	0,002425
Cluster 1	biological_process	0030001 metal ion transport	114207/Ryr1;116601/Atp2a1;140727/Ca	22	272	585	17276	9,210465	0,080882	7,75E-06	4,321434	0,000153	0,5	0,002474
Cluster 1	biological_process	0016310 phosphorylation	116671/Cdk5r1;117558/Mylk2;24183/Ak	33	272	1070	17276	16,84649	0,121324	2,10E-05	4,095711	0,000168	0,5	0,002713
Cluster 1	biological_process	0003300 cardiac muscle hypertrophy	117505/Csrp3;29557/Myh7;363925/Myl2	5	272	35	17276	0,551053	0,018382	7,38E-10	6,046919	0,000206	0,5	0,003223
Cluster 1	biological_process	0051602 response to electrical stimulus	140939/Trim63;24799/Eef1a2;29148/My	7	272	77	17276	1,212318	0,025735	5,48E-08	5,310066	0,000209	0,5	0,003256
Cluster 1	biological_process	0032787 monocarboxylic acid metabolic process	116676/Aldh1a2;116719/Acacb;24534/Lc	18	272	439	17276	6,911785	0,066176	8,31E-06	4,306151	0,000217	0,5	0,003375
Cluster 1	biological_process	0032844 regulation of homeostatic process	114207/Ryr1;170546/Ryr3;245956/Scn3b	20	272	521	17276	8,202825	0,073529	1,24E-05	4,215792	0,000234	0,5	0,003617
Cluster 1	biological_process	0086001 cardiac muscle cell action potential	245956/Scn3b;24907/Dmd;25399/Cacna	5	272	36	17276	0,566798	0,018382	1,41E-09	5,941415	0,000236	0,5	0,003622
Cluster 1	biological_process	0006110 regulation of glycolytic process	171009/Actn3;25747/Ppara;29148/Myog	5	272	36	17276	0,566798	0,018382	1,41E-09	5,941415	0,000236	0,5	0,003622
Cluster 1	biological_process	0050877 nervous system process	113995/P2rx5;24245/Camk2b;24261/Chr	15	272	2366	17276	37,25122	0,055147	0,999962	-3,955485	0,999991	0,963626156	0,996354
Cluster 1	biological_process	0060341 regulation of cellular localization	114906/Pkia;116671/Cdk5r1;117558/My	29	272	909	17276	14,31165	0,106618	2,90E-05	4,020674	0,000244	0,5	0,003663
Cluster 1	biological_process	0010611 regulation of cardiac muscle hypertrophy	140939/Trim63;29161/Cav3;494021/Lmc	6	272	56	17276	0,881686	0,022059	1,87E-08	5,503115	0,000242	0,5	0,003663
Cluster 1	biological_process	0043271 negative regulation of ion transport	291245/Actn2;29161/Cav3;29209/Casq2	10	272	162	17276	2,55059	0,036765	1,16E-06	4,723684	0,000251	0,5	0,003715
Cluster 1	biological_process	0051494 negative regulation of cytoskeleton organization	24851/Tpm1;25566/Tmod1;29161/Cav3;	9	272	133	17276	2,094003	0,033088	6,86E-07	4,828913	0,000259	0,5	0,00381
Cluster 1	biological_process	1904064 positive regulation of cation transmembrane transport	24907/Dmd;25399/Cacna2d1;291245/Ac	9	272	134	17276	2,109748	0,033088	7,93E-07	4,800033	0,000274	0,5	0,003998
Cluster 1	biological_process	0032956 regulation of actin cytoskeleton organization	116671/Cdk5r1;24851/Tpm1;25566/Tmo	14	272	299	17276	4,707571	0,051471	6,66E-06	4,354671	0,000292	0,5	0,004195
Cluster 1	biological_process	0051283 negative regulation of sequestering of calcium ion	114207/Ryr1;170546/Ryr3;29209/Casq2;	6	272	58	17276	0,913174	0,022059	3,84E-08	5,374446	0,000294	0,5	0,004195
Cluster 1	biological_process	2001259 positive regulation of cation channel activity	25399/Cacna2d1;291245/Actn2;296345/	6	272	61	17276	0,960408	0,022059	1,04E-07	5,192413	0,000387	0,5	0,005393
Cluster 1	biological_process	0010883 regulation of lipid storage	116719/Acacb;25747/Ppara;290223/Fitm	5	272	40	17276	0,629775	0,018382	1,37E-08	5,557094	0,000391	0,5	0,005403
Cluster 1	biological_process	0050850 positive regulation of calcium-mediated signaling	296345/Jph2;494021/Lmcd1;59299/Trdn	5	272	41	17276	0,64552	0,018382	2,26E-08	5,46929	0,00044	0,5	0,00601
Cluster 1	biological_process	0051281 positive regulation of release of sequestered calcium ion into cytosol	29155/Capn3;296345/Jph2;59299/Trdn;(	5	272	41	17276	0,64552	0,018382	2,26E-08	5,46929	0,00044	0,5	0,00601

Cluster	Category	Term	Entry	r	R	n	N	rEXP	r/R	p(z)	z	p	p(hommel)	p(BH)
Cluster 1	biological_process	0051289 protein homotetramerization	114207/Ryr1;116719/Acacb;25630/Pkm;	7	272	87	17276	1,369762	0,025735	5,84E-07	4,861095	0,000444	0,5	0,006044
Cluster 1	biological_process	0006796 phosphate-containing compound metabolic process	116671/Cdk5r1;117533/Gmpr;117558/M	46	272	1781	17276	28,04075	0,169118	0,000153	3,609547	0,00052	0,5	0,006984
Cluster 1	biological_process	0045445 myoblast differentiation	27064/Ankrd1;29148/Myog;299266/Asb	5	272	43	17276	0,677009	0,018382	5,72E-08	5,302271	0,00055	0,5	0,007343
Cluster 1	biological_process	0051928 positive regulation of calcium ion transport	25399/Cacna2d1;25400/Camk2a;29155/(	8	272	121	17276	1,905071	0,029412	3,97E-06	4,466557	0,000661	0,5	0,008733
Cluster 1	biological_process	0007268 chemical synaptic transmission	113995/P2rx5;117558/Mylk2;192649/Pr	15	272	364	17276	5,730956	0,055147	4,00E-05	3,944382	0,000686	0,5	0,009003
Cluster 1	biological_process	0006163 purine nucleotide metabolic process	117533/Gmpr;24183/Ak1;24583/Myh3;2	14	272	328	17276	5,164158	0,051471	3,80E-05	3,956799	0,000733	0,5	0,009393
Cluster 1	KEGG_DISEASE	Hypertrophic cardiomyopathy (HCM) (H00292)	117505/Csrp3;24585/Myl3;24837/Tnnt2;	9	93	18	3760	0,445213	0,096774	5,22E-39	13,01212	9,50E-11	1,28E-08	1,06E-08
Cluster 1	KEGG_DISEASE	Nemaline myopathy (H00698)	117537/Klhl41;171409/Tnnt1;29437/Act	7	93	9	3760	0,222606	0,075269	2,48E-48	14,56113	1,56E-10	2,11E-08	1,06E-08
Cluster 1	KEGG_DISEASE	Dilated cardiomyopathy (DCM) (H00294)	117505/Csrp3;171009/Actn3;24837/Tnn	11	93	42	3760	1,03883	0,11828	1,25E-23	9,950802	2,65E-09	3,56E-07	1,20E-07
Cluster 1	KEGG_DISEASE	Distal arthrogryposis (DA) (H00811)	24583/Myh3;24838/Tnnt3;252942/Myh8	6	93	16	3760	0,395745	0,064516	7,91E-20	9,038931	1,28E-06	0,00016996	4,34E-05
Cluster 1	KEGG_DISEASE	Muscle glycogen storage disease (H01762)	24534/Ldhb;24645/Pgm1;24701/Pygm;2	7	93	30	3760	0,742021	0,075269	7,63E-14	7,384925	5,78E-06	0,000756783	0,000157
Cluster 1	KEGG_DISEASE	Limb-girdle muscular dystrophy (LGMD) (H00593)	29155/Capn3;29161/Cav3;303468/Sgca;3	7	93	38	3760	0,939894	0,075269	1,00E-10	6,361029	3,05E-05	0,003839157	0,000518
Cluster 1	KEGG_DISEASE	Glycogen storage diseases (GSD) (H00069)	24534/Ldhb;24645/Pgm1;24701/Pygm;2	7	93	40	3760	0,989362	0,075269	3,85E-10	6,151007	4,32E-05	0,005403881	0,000653
Cluster 1	KEGG_PATHWAY	Hypertrophic cardiomyopathy (HCM) (KEGG:05410)	140727/Cacng6;24585/Myl3;24837/Tnnt	19	115	81	8400	1,108929	0,165217	1,60E-66	17,18922	6,34E-19	1,38E-16	1,38E-16
Cluster 1	KEGG_PATHWAY	Dilated cardiomyopathy (DCM) (KEGG:05414)	140727/Cacng6;24585/Myl3;24837/Tnnt	18	115	86	8400	1,177381	0,156522	8,78E-56	15,69052	5,14E-17	1,11E-14	5,58E-15
Cluster 1	KEGG_PATHWAY	Cardiac muscle contraction (KEGG:04260)	140727/Cacng6;24585/Myl3;24837/Tnnt	17	115	78	8400	1,067857	0,147826	3,88E-55	15,59592	1,94E-16	4,16E-14	1,05E-14
Cluster 1	KEGG_PATHWAY	Adrenergic signaling in cardiomyocytes (KEGG:04261)	140727/Cacng6;24245/Camk2b;24585/M	21	115	142	8400	1,944048	0,182609	4,27E-44	13,87864	1,89E-16	4,04E-14	1,05E-14
Cluster 1	KEGG_PATHWAY	Arrhythmogenic right ventricular cardiomyopathy (ARVC) (KEGG:05412)	140727/Cacng6;171009/Actn3;25399/Ca	13	115	70	8400	0,958333	0,113043	8,24E-36	12,43692	7,35E-12	1,57E-09	3,19E-10
Cluster 1	KEGG_PATHWAY	Glucagon signaling pathway (KEGG:04922)	116719/Acacb;24245/Camk2b;24534/Ldl	11	115	101	8400	1,382738	0,095652	5,92E-17	8,284681	1,10E-07	2,34E-05	3,99E-06
Cluster 1	KEGG_PATHWAY	Oxytocin signaling pathway (KEGG:04921)	114207/Ryr1;117558/Mylk2;140727/Cac	12	115	156	8400	2,135714	0,104348	3,44E-12	6,860129	1,31E-06	0,000275848	4,05E-05
Cluster 1	KEGG_PATHWAY	Olfactory transduction (KEGG:04740)	24245/Camk2b;25400/Camk2a	2	115	1312	8400	17,9619	0,017391	0,999982	-4,128135	0,999999	0,99984269	0,999843
Cluster 1	KEGG_PATHWAY	Calcium signaling pathway (KEGG:04020)	113995/P2rx5;114207/Ryr1;116601/Atp2	11	115	189	8400	2,5875	0,095652	5,02E-08	5,325913	5,19E-05	0,010898741	0,001408
Cluster 1	KEGG_PATHWAY	Apelin signaling pathway (KEGG:04371)	114207/Ryr1;117558/Mylk2;170546/Ryr3	9	115	141	8400	1,930357	0,078261	1,19E-07	5,166805	0,000128	0,026650813	0,003075
Cluster 1	KEGG_PATHWAY	Glycolysis / Gluconeogenesis (KEGG:00010)	24534/Ldhb;24645/Pgm1;24959/Pgam2;	6	115	71	8400	0,972024	0,052174	1,26E-07	5,156624	0,000401	0,082618658	0,008703
Cluster 1	molecular_function	0003779 actin binding	100910104/Xirp1;116653/Cap2;117028/I	46	268	372	16607	6,003252	0,171642	1,66E-62	16,6445	1,46E-27	1,20E-24	6,02E-25
Cluster 1	molecular_function	0008307 structural constituent of muscle	114108/Pdlim3;117505/Csrp3;24584/My	13	268	27	16607	0,43572	0,048507	1,69E-82	19,20468	6,17E-17	5,08E-14	1,70E-14
Cluster 1	molecular_function	0051015 actin filament binding	117028/Bin1;171009/Actn3;24583/Myh3	23	268	157	16607	2,53363	0,085821	4,46E-39	13,02422	7,78E-16	6,39E-13	1,60E-13
Cluster 1	molecular_function	0005515 protein binding	100910104/Xirp1;113906/Hspb8;114108	171	268	6910	16607	111,512	0,63806	5,34E-14	7,432161	1,34E-13	1,10E-10	2,22E-11
Cluster 1	molecular_function	0031432 titin binding	140939/Trim63;27064/Ankrd1;291245/A	7	268	9	16607	0,14524	0,026119	8,00E-74	18,13789	9,23E-12	7,57E-09	1,27E-09
Cluster 1	molecular_function	0005523 tropomyosin binding	171409/Tnnt1;24837/Tnnt2;24838/Tnnt3	7	268	15	16607	0,242067	0,026119	6,05E-44	13,85359	1,52E-09	1,24E-06	1,57E-07
Cluster 1	molecular_function	0051371 muscle alpha-actinin binding	306616/Myom2;307982/Nrap;309760/M	7	268	15	16607	0,242067	0,026119	6,05E-44	13,85359	1,52E-09	1,24E-06	1,57E-07

Cluster	Category	Term	Entry	r	R	n	N	rEXP	r/R	p(z)	z	p	p(hommel)	p(BH)
Cluster 1	molecular_function	0042805 actinin binding	114108/Pdlim3;117505/Csrp3;306616/M	10	268	45	16607	0,7262	0,037313	2,23E-28	10,98598	1,97E-09	1,61E-06	1,80E-07
Cluster 1	molecular_function	0003676 nucleic acid binding	100910104/Xirp1;24799/Eef1a2;24890/E	21	268	3626	16607	58,51557	0,078358	1	-5,592257	1	0,999999612	1
Cluster 1	molecular_function	0004930 G-protein coupled receptor activity	192649/Prokr2;363866/Rxfp2;58965/Rar	5	268	1868	16607	30,14536	0,018657	1	-4,900936	1	0,999996282	0,999999
Cluster 1	molecular_function	0032403 protein complex binding	117028/Bin1;171009/Actn3;24583/Myh3	39	268	906	16607	14,62082	0,145522	1,92E-11	6,610508	2,01E-08	1,64E-05	1,65E-06
Cluster 1	molecular_function	0043167 ion binding	113995/P2rx5;114108/Pdlim3;114207/Ry	121	268	5051	16607	81,51189	0,451493	6,26E-08	5,285882	2,18E-07	0,000177426	1,50E-05
Cluster 1	molecular_function	0005509 calcium ion binding	114207/Ryr1;116601/Atp2a1;116671/Cd	27	268	635	16607	10,24749	0,100746	3,73E-08	5,379687	4,56E-06	0,003644903	0,000209
Cluster 1	molecular_function	0005198 structural molecule activity	114108/Pdlim3;117505/Csrp3;161476/H	28	268	678	16607	10,94141	0,104478	5,52E-08	5,308571	5,23E-06	0,004174851	0,000227
Cluster 1	molecular_function	0042166 acetylcholine binding	24261/Chrnb1;25753/Chrng;54240/Chrn	5	268	17	16607	0,274342	0,018657	4,50E-20	9,100352	5,57E-06	0,004447866	0,00023
Cluster 1	molecular_function	0044325 ion channel binding	171009/Actn3;245956/Scn3b;25177/Fhl1	11	268	131	16607	2,114048	0,041045	3,09E-10	6,185659	9,04E-06	0,007204587	0,000339
Cluster 1	molecular_function	0005261 cation channel activity	113995/P2rx5;114207/Ryr1;140727/Cacr	17	268	307	16607	4,954296	0,063433	1,83E-08	5,506971	1,08E-05	0,008577241	0,000387
Cluster 1	molecular_function	0046872 metal ion binding	113995/P2rx5;114108/Pdlim3;114207/Ry	77	268	3009	16607	48,55856	0,287313	2,72E-06	4,547226	1,21E-05	0,009658064	0,0004
Cluster 1	molecular_function	0022836 gated channel activity	113995/P2rx5;114207/Ryr1;140727/Cacr	17	268	315	16607	5,083399	0,063433	3,73E-08	5,379644	1,50E-05	0,011961892	0,000477
Cluster 1	molecular_function	0015085 calcium ion transmembrane transporter activity	114207/Ryr1;116601/Atp2a1;140727/Ca	10	268	127	16607	2,049497	0,037313	9,53E-09	5,620277	4,04E-05	0,031822435	0,00108
Cluster 1	molecular_function	0003723 RNA binding	100910104/Xirp1;24799/Eef1a2;25630/P	8	268	1632	16607	26,33685	0,029851	0,999926	-3,793366	0,999987	0,989243247	0,997859
Cluster 1	molecular_function	0004888 transmembrane signaling receptor activity	113995/P2rx5;114207/Ryr1;170546/Ryr3	16	268	2350	16607	37,92377	0,059701	0,999946	-3,873564	0,999984	0,987230901	0,997859
Cluster 1	molecular_function	0017080 sodium channel regulator activity	245956/Scn3b;29161/Cav3;362242/Snta1	5	268	34	16607	0,548684	0,018657	6,62E-10	6,064457	0,0002	0,153811106	0,004715
Cluster 1	molecular_function	0022890 inorganic cation transmembrane transporter activity	114207/Ryr1;116601/Atp2a1;140727/Ca	21	268	547	16607	8,827362	0,078358	1,33E-05	4,200121	0,000226	0,173687055	0,00491
Cluster 1	molecular_function	0099094 ligand-gated cation channel activity	114207/Ryr1;170546/Ryr3;24261/Chrnb1	7	268	76	16607	1,226471	0,026119	6,90E-08	5,267792	0,000223	0,171258462	0,00491
Cluster 1	molecular_function	0005516 calmodulin binding	114207/Ryr1;117558/Mylk2;24245/Caml	10	268	160	16607	2,582044	0,037313	1,46E-06	4,676538	0,000276	0,209217561	0,005693
Cluster 1	molecular_function	0016301 kinase activity	113906/Hspb8;116671/Cdk5r1;117558/M	27	268	808	16607	13,03932	0,100746	3,22E-05	3,996038	0,000284	0,214958673	0,005721
Cluster 1	molecular_function	0015075 ion transmembrane transporter activity	113995/P2rx5;114207/Ryr1;116601/Atp2	28	268	856	16607	13,81393	0,104478	3,89E-05	3,951069	0,000309	0,232528743	0,005994
Cluster 1	molecular_function	0005262 calcium channel activity	114207/Ryr1;140727/Cacng6;170546/Ry	8	268	106	16607	1,710604	0,029851	5,77E-07	4,86345	0,000319	0,239552714	0,005994
Cluster 1	molecular_function	0005245 voltage-gated calcium channel activity	114207/Ryr1;25399/Cacna2d1;29658/Ca	5	268	42	16607	0,677786	0,018657	5,81E-08	5,299433	0,00055	0,392484254	0,009083
Cluster 1	molecular_function	0017022 myosin binding	117558/Mylk2;24907/Dmd;25028/Ampd	6	268	64	16607	1,032817	0,022388	3,97E-07	4,936925	0,000572	0,404281988	0,00925

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Supplementary material to manuscript III

Manuscript III – Supplemental material

Schwartz C. L., Svingen T., Christiansen S., Johansson, H. K. L., Pedersen M., Frandsen H. L., Frederiksen K. A., Govers L. C., Pask A. J. and Vinggaard A. M. Expression of estrogen and Wnt related factors in the rat perineal tissues during normal development and following disruption of fetal androgen action. *Manuscript in preparation*

Contents:

Supplementary Table 1

Table S1 – Chemical concentrations

Group	**Final concentrations in media**	
Control female	EtOH 0.05%	DMSO 0.05%
DHT	DHT 1nm	DMSO 0.05%
Enz1	DHT 1nm	Enzalutamide 1µM
Enz100	DHT 1nm	Enzalutamide 100µM
Vin1	DHT 1nm	Vinclozolin 1µM
Vin100	DHT 1nm	Vinclozolin 100µM